

**TOXINOLOGICAL CHARACTERIZATIONS
OF THE VENOM OF HUMP-NOSED PIT VIPER
(*HYPNALE HYPNALE*)**

TAN CHOO HOCK

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Name of Candidate: TAN CHOO HOCK

(I.C/Passport No: 810206-07-5353)

Registration/Matric No: MHA 090027

Name of Degree: DOCTOR OF PHILOSOPHY

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(HYPNALE HYPNALE)**

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(No. K.P/Pasport: **810206-07-5353**)

No. Pendaftaran/Matrik: **MHA 090027**

Nama Ijazah: **DOKTOR FALSAFAH**

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Abstract

Hump-nosed pit viper (*Hypnale hypnale*) is a medically important snake in Sri Lanka and Western Ghats of India. Envenomation by this snake still lacks effective antivenom clinically. The species is also often misidentified, resulting in inappropriate treatment.

The median lethal dose (LD₅₀) of *H. hypnale* venom varies from 0.9 µg/g intravenously to 13.7 µg/g intramuscularly in mice. The venom shows procoagulant, hemorrhagic, necrotic, and various enzymatic activities including those of proteases, phospholipases A₂ and L-amino acid oxidases which have been partially purified. The monovalent Malayan pit viper antivenom and Hemato polyvalent antivenom (HPA) from Thailand effectively cross-neutralized the venom's lethality *in vitro* (median effective dose, ED₅₀ = 0.89 and 1.52 mg venom/mL antivenom, respectively) and *in vivo* in mice, besides the procoagulant, hemorrhagic and necrotic effects. HPA also prevented acute kidney injury in mice following experimental envenomation. Therefore, HPA may be beneficial in the treatment of *H. hypnale* envenomation.

H. hypnale-specific antiserum and IgG, produced from immunization in rabbits, effectively neutralized the venom's lethality and various toxicities, indicating the feasibility to produce an effective specific antivenom with a common immunization regime. On indirect ELISA, the IgG cross-reacted extensively with Asiatic crotalid venoms, particularly that of *Calloselasma rhodostoma* (73.6%), suggesting that the two phylogenically related snakes share similar venoms antigenic properties. Double-sandwich ELISA was specific and able to distinguish and quantify venoms of *H. hypnale*, *Daboia russelii* and *Echis carinatus sinhaleyus* (three common Sri Lankan viperids) in human sera; hence it may be useful in diagnostics and venom level monitoring especially during clinical studies.

In rabbits, the venom when injected intravenously showed a rapid distribution phase ($t_{1/2\alpha} = 0.6$ h) and a slow elimination phase ($t_{1/2\beta} = 20$ h), consistent with prolonged abnormal hemostasis reported. The intramuscular bioavailability was exceptionally low ($F_{i.m} = 4\%$), accountable for the highly varied LD_{50} between intravenous and intramuscular envenomings in animals. HPA infused post-envenomation markedly reduced the serum venom levels and subsequently induced venom redistribution. The redistributed venom was completely neutralized by a second dose of HPA.

Proteomic study by shotgun-liquid chromatography-mass spectrometry/mass spectrometry (shotgun-LC-MS/MS) revealed 52 proteins in the venom, 70% of which are toxinologically related. The combined use of reverse-phase high performance liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, peptide sequencing and mass fingerprinting technologies enabled the identification of the venom major components, i.e. zinc-dependent metalloproteases, phospholipases A₂, L-amino acid oxidases, serine proteases and C-type lectins. These toxins correlate with the venom's principal effects: hemotoxicity (hemorrhage, coagulopathy) and tissue destruction (necrosis). Understanding of the venom composition is essential for ascertaining the principal toxins, and for optimizing antivenom formulation. Furthermore, hypnabin, a 37 kDa thrombin-like enzyme was purified. It exhibits arginine esterase and amidase activities, besides distinct specificity towards different mammalian fibrinogens. In fibrinogen coagulation, hypnabin predominantly releases fibrinopeptide A, followed slowly by a small amount of fibrinopeptide B. The findings provide insights into the structures and mechanism of the thrombin-like enzyme, which are important for elucidation of the pathophysiology and for potential drug discovery, e.g. an anticoagulant for thromboembolic disorders.

Abstrak

Ular *Hypnale hypnale* adalah penting dari segi perubatan di Sri Lanka dan Western Ghats, India. Envenomasi (patukan berbisa) ular ini masih tiada penawar bisa yang berkesan. Species ini juga kerap salah dikenalpasti dan ini mengakibatkan rawatan yang tidak sewajar.

Dos maut median (LD₅₀) bisa *H. hypnale* berbeza dari 0.9 µg/g secara intravena ke 13.7 µg/g secara intramuskular pada tikus. Bisa ini menunjukkan kesan-kesan prokoagulan, pendarahan, nekrosis dan aktiviti pelbagai enzim termasuk proteases, phospholipases A₂ serta L-amino acid oxidases yang separanya telah diekstrakkan. Monovalent Malayan pit viper antivenom dan Hemato polyvalent antivenom (HPA) dari Thailand meneutralkan kesan maut bisa ini secara *in vitro* (dos efektif median, ED₅₀ = 0.89 dan 1.52 mg bisa/mL antivenom, masing-masing) dan *in vivo* pada tikus, serta kesan-kesan prokoagulan, pendaraha dan nekrosis. HPA selepas envenomasi eksperimen turut mencegah kecederaan akut ginjal pada tikus. Maka, HPA mungkin bermanfaat sebagai rawatan bagi envenomasi *H. hypnale*.

Antiserum dan IgG spesifik kepada *H. hypnale* yang dihasilkan melalui imunisasi arnab meneutralkan kesan-kesan maut dan toksik bisa itu. Ini menunjukkan kebolehkaksanaan penghasilan suatu penawar bisa spesifik dengan rejim imunisasi yang am. Kajian 'indirect ELISA' menunjukkan IgG itu bereaksi-silang secara ekstensif dengan pelbagai bisa ular jenis 'crotalid' di Asia, khususnya *Calloselasma rhodostoma* (73.6%). Ini menunjukkan kedua-dua ular yang berhubungan filogenetik ini berkongsi antigen-antigen bisa yang sama. 'Double-sandwich ELISA' adalah spesifik dan dapat membezakan serta mengukur paras bisa-bisa *H. hypnale*, *Daboia russelii* dan *Echis carinatus sinhaleyus* (tiga ular jenis viperid yang biasa di Sri Lanka) dalam serum

manusia. Ini menunjukkan potensinya digunakan untuk diagnostik dan pemantauan paras bisa khususnya semasa kajian klinikal.

Bisa ini apabila disuntik secara intravena pada arnab menunjukkan fasa 'distribution' yang pantas ($t_{1/2\alpha} = 0.6$ h) dan fasa 'elimination' yang lambat ($t_{1/2\beta} = 20$ h), konsisten dengan hemostasis abnormal berpanjangan seperti dilaporkan. 'Intramuscular bioavailability' bisa ini adalah amat rendah ($F_{i.m} = 4\%$), dan menjelaskan perbezaan LD_{50} yang besar antara cara pemberian intravena dan intramuskular pada haiwan. Infusi HPA selepas envenomasi eksperimental mengurangkan paras bisa dengan banyak dan seterusnya mendorong 'venom redistribution'. Bisa yang diedar semula itu dineutralkan oleh dos kedua HPA.

Kajian proteomic dengan 'shotgun-liquid chromatography-mass spectrometry/mass spectrometry (shotgun-LC-MS/MS)' mengenalpasti 52 protein dalam bisa, di mana 70% berkaitan dengan aktiviti toksin. Penggunaan 'reverse-phased high performance liquid chromatography', 'sodium dodecyl sulfate-polyacrylamide gel electrophoresis', 'peptide sequencing' bersama 'mass fingerprinting' membolehkan komponen-komponen utama bisa itu dikenalpastikan, iaitu zinc-dependent metalloproteases, phospholipases A_2 , L-amino acid oxidases, serine proteases dan C-type lectins. Toksin-toksin ini berkorelasi dengan kesan-kesan utama bisa ini: hematoksik (pendarahan, koagulopati) dan kemusnahan tisu (nekrosis). Pemahaman komposisi bisa adalah penting bagi menentupasti toksin-toksin utama, dan untuk mengoptimumkan formulasi penawar bisa. Tambahan, hypnabin, suatu enzim jenis trombin berjisim 37 kDa telah diekstrakkan. Hypnabin menunjukkan aktiviti-aktiviti arginine ester hydrolase dan amidase, serta spesifikasi yang berbeza terhadap fibrinogen mamalia yang berlainan. Bagi koagulasi fibrinogen, hypnabin membebaskan fibrinopeptide A kebanyakannya,

diikuti amaun kecil fibrinopeptide B yang dibebaskan dengan lambat. Penemuan sedemikian memberi pemahaman terhadap struktur dan mekanisme thrombin-like enzyme ini, penting bagi penerangan patofisiologi dan penemuan ubat-ubatan, contohnya, sebagai antikoagulan bagi rawatan penyakit tromboembolik.

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List of Symbols and Abbreviations

approx.	approximately	HRP	horseradish peroxidase
%	percent	HCl	hydrochloric acid
®	registered	H ₂ SO ₄	sulfuric acid
™	trademark	IgG	immunoglobulin G
μg	microgram	<i>i.m.</i>	intramuscular
μL	microliter	<i>i.p.</i>	intraperitoneal
μm	micrometer	<i>i.v.</i>	<i>intravenous</i>
μmole	micromole	kg	kilogram
°C	degree Celsius	kDa	kilo-Dalton
3-FTX	three-finger toxin	<i>k_{cat}</i>	catalytic constant
Abs	Absorbance	K _m	Michaelis constant
AKI	Acute kidney injury	LAAO	L-amino acid oxidase
AUC	area under the curve	LC-MS	liquid chromatography-mass spectrometry
C ₀	Concentration at t ₀	LD ₅₀	median lethal dose
CI	confidence interval	m	meter
CL	systemic clearance by body weight	M	molar
cm	centimeter	MCD	minimum coagulant dose
CTL	C-type lectin	mg	milligram
Da	Dalton	mL	milliliter
HPA	Hemato polyvalent antivenom	MLD	minimum lethal dose
HPLC	high performance liquid chromatography	mm	millimeter

mM	millimolar	PLA ₂	phospholipase A ₂
mmole	millimole	SDS	sodium dodecyl sulfate
MND	minimum necrotic dose	SVMP	snake venom metalloprotease
MS	mass spectrometry	SVSP	snake venom serine protease
MPVA	monovalent Malayan pit viper antivenom	TLE	thrombin-like enzyme
mya	million years ago	V	volt
n	number of (subject)	v/v	volume/volume
NaCl	sodium chloride	w/v	weight/volume
ng	nanogram	nm	nanometer
nmole	nanomole	s	second
OPD	ortho-phenylenediamine	S.E.M.	standard error of the mean
P	potency	t _{1/2α}	half-life at distribution phase
<i>p</i>	<i>p</i> value	t _{1/2β}	half-life at elimination phase
PAGE	polyacrylamide gel electrophoresis	TEMED	tetramethylethylenediamine
PBS	phosphate-buffered saline	u	atomic mass unit (a.m.u.)
PCR	polymerase chain reaction	V _c	volume of central compartment
PDE	phosphodiesterase	V _{D(area)}	volume of distribution by area
		(V _D) _{ss}	apparent volume of distribution at steady state

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CHAPTER 1

Introduction

1.1 Snakes and humans

The relationship between snakes and humans has been complicated but amazing from prehistorical times to modern days. Evolutionary arms race begun early by constrictors was followed by venomous snakes throughout the mammalian evolution. The consequence was particularly significant in the anthropoid and primate evolution (Isbell, 2006): a fear circuit independent of conscious cognition that lies heavily on the limbic structures (Öhman and Mineka, 2003), selective pressures for visual pathways adaptation and further brain expansion that became unique to primates that included primitive humans (Shibasaki and Kawai, 2009; Hayakawa *et al.*, 2011). The influence of snakes continued in ancient cultures. Historical representations of snakes often reflected an ignorance of their true nature, demonstrating a veritable dichotomy between the Good and Evil: at times revered as virtuous power of knowledge, protection and productivity; at other times reviled as evil and cause of fall. In modern age, similar conflict exists: snakes' destructive power was greatly feared for, but as civilization progressed snakes became animals that were hunted and farmed for purposes like show, food, traditional medicines, leather production, and biological control. Interestingly, the venomous character of certain species contributes toward their medical significance in human lives, also in a paradoxical context: pathologically, venom is viewed as a source of sickness and death; therapeutically, as a source of novel drug discovery (Chippaux, 2006; Koh *et al.*, 2006).

1.2 Snake bites and envenomation: South Asia

Venomous bites (envenomation) by snakes constitute a substantial health problem especially in the tropics and subtropics (Chippaux, 1998; Kasturiratne *et al.*, 2008). The

complications, acute or chronic, include psychological trauma, organ dysfunctions, permanent disability, and death. It is hence duly indexed as a classifiable “disease” according to the WHO International Classification of Disease (ICD-10), appropriately categorized as a Neglected Tropical Disease (WHO, 2009), and aptly described as a disease of poverty (Harrison *et al.*, 2009). In spite of these, envenoming issues have been continuously neglected systemically in many parts of the world (Gutiérrez *et al.*, 2006; Alirol *et al.*, 2010), posing a great challenge to the World health Organization in its promotion of ‘Health for All’.

At least 3 million envenomations occur worldwide (Chippaux, 2006), and the highest annual number (121,000) came from South Asia (Kasturiratne *et al.*, 2008), with India being the most affected region recording the highest snakebite mortality rate in the world: 35,000-50,000 deaths occur yearly based on WHO direct estimates (Chippaux, 1998; Kasturiratne *et al.*, 2008). Epidemiological studies indicated that it is an occupational health hazard affecting particularly farmers and other agricultural workers, although frequent bites among housewives and students had also been reported (Alirol *et al.*, 2010). In certain occasions e.g. the 2007 monsoon flood in Bangladesh, snakebites even became the second commonest cause of death after drowning, illustrating how this problem can be as serious as the other tropical health issues in the region (WHO, 2007). In anticipation of climatic changes due to global warming, snakebite “outbreak” that comes with flash flood is no longer a fictional scene, therefore needing precautionary measures that include antivenom stock-up and adequate training of the medical personnel in managing snakebite emergency from time to time. A recent example was shown in 2011 Thailand monsoon flood, where the Thai Ministry of Public Health boosted the stockpile of antivenoms by a further 3,500 dosages and dispatched a few hundred to flood-hit areas, ensuring every public hospital outside

Bangkok has 10 dosages in its stock in anticipation of increased snakebite emergency. Such policy could only be possible when a well-supported national antivenom manufacturing facility is available with readily sufficient products and efficient distribution system within the country, as shown in the Thai scenario. The practicality of such recommendation in economically diverse areas in South Asia unfortunately remains suboptimistic. Long-term and systematic efforts that include intensive research and upgraded antivenom management are therefore very much needed for this region.

There are at least 67 front-fanged venomous snakes in South Asia with wide varieties across different areas. Sri Lanka and Southwestern India share similar biogeography and most of the venomous species concerned. For decades, deadly venomous snakes prevalent in the region were thought to mainly consist of the Big Four: the Indian cobra (*Naja naja*), the krait (*Bungarus caeruleus*) (and *B. ceylonicus* in Sri Lanka), the Russell's viper (*Daboia russelii*) and the saw-scaled viper (*Echis carinatus*). Envenomation by these snakes were identified based on clinical history and syndromes; while the definite treatment has been conventionally the polyvalent antivenom raised against venoms of the Big Four, produced by Indian manufacturers (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2009; Alirol *et al.*, 2010). Nevertheless, like the countries in Africa and Latin America, the management of snakebite envenomation in South Asia faces challenges such as patient access to healthcare, dissemination of knowledge and education for public and medical personnel, improvement of diagnostic methods, and improvement of the efficacy, species coverage as well as distribution of antivenoms (Gutiérrez *et al.*, 2006; Chippaux, 2008; Alirol *et al.*, 2010). In addition, the properties of many snake venoms, especially related to toxin variations, awaits further exploratory studies for a better understanding of the pathophysiology and for finding a solution to the failure of antivenom therapy in some cases. The emergence or re-identification of

some venomous species which were previously overlooked, due to a lack of taxonomy knowledge or misidentification, is another pertinent issue relevant to snake envenomation in this region, as exemplified by the hump-nosed pit viper, *Hypnale hypnale* (Joesph *et al.*, 2007; Ariaratnam *et al.*, 2008).

1.3 Envenomation by *Hypnale hypnale*

In Sri Lanka and Western Ghats of India, bites from the hump-nosed pit viper (*Hypnale hypnale*) have recently been authenticated as the leading cause of snakebites with severe complications: local tissue destruction, hemostatic dysfunction (coagulopathy, fibrinolysis, thrombocytopenia, spontaneous systemic hemorrhage) as well as acute kidney injury, with an overall fatality rate of 1.7% (Joesph *et al.*, 2007; Ariaratnam *et al.*, 2008). The authentication that confirmed the emergence of *H. hypnale* as a snake of medical importance capable of lethal envenomation has rendered the earlier “Big Four” concept obsolete in terms of its completeness. Sound clinical and epidemiological studies are also restricted due to ambiguous venomous status and frequent misidentification of the snake (Simpson and Norris, 2007; Alirol *et al.*, 2010).

Despite the severity and urgency of the problem, there is no species-specific antivenom ever produced against *H. hypnale* envenoming; while the existing antivenoms available in the region were all proven ineffective clinically (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008). Patients envenomed by *H. hypnale* are therefore given supportive and ancillary treatment including amputation for unsalvageable necrotic limb that adds to the toll of human suffering. Other severe complications such as acute kidney injury and delayed extensive hemorrhages (Ariaratnam *et al.*, 2008; Sunanda *et al.*, 2010) further complicate the clinical management. Besides, the problem of *H. hypnale* envenoming is

further confounded by inaccurate diagnosis of the biting species, very often as *Echis carinatus* or *Daboia russelii*. This results in suboptimal syndrome monitoring and unwarranted antivenom therapy that exposes patients to fatal adverse reactions, besides incurring high cost of the antivenom. The root of the problem regarding *H. hypnale* envenomation hence appears to be a gap in the research knowledge of the venom biomedical properties (that can help elucidate the envenoming pathophysiology and improve diagnostics), a lack of effort in the search for an effective antidote for the envenomation, in addition to confusion of the snake taxonomy and systematics.

1.4 Objectives of the study

A research project on the toxinological profiles of *H. hypnale* venom, with a problem-based and solution-oriented approach, is therefore relevant to holistically address the envenoming issue. The current study endeavored (i) to elucidate the toxinological properties of the venom via biochemical assays coupled with *in vitro* and *in vivo* animal models, (ii) to search for effective antidotes, (iii) to enhance the diagnostics of the biting species, and (iv) to demonstrate the feasibility of a specific antivenom production. The specific objectives are summarized as follows:

1. To study the toxinological properties of *Hypnale hypnale* venom and its fractionated components, using biochemical assays and animal models.
2. To establish the lethal and toxic parameters for *H. hypnale* venom and to study the neutralizing efficacy of selected antivenoms, using both *in vitro* and *in vivo* approaches.

3. To examine the neutralization of nephrotoxic effect of *H. hypnale* venom by a paraspecific antivenom in a mouse model.
4. To study the immunogenicity of *H. hypnale* venom in rabbits for the production of polyclonal antibody raised against the venom.
5. To assess the feasibility of the production of *H. hypnale*-specific antivenom at laboratory setting.
6. To develop a diagnostic assay for the identification of biting snakes (*H. hypnale*, *Echis carinatus*, *Daboia russelii*) using immunoassay.
7. To study the pharmacokinetics of *H. hypnale* venom and changes effected by the use of a paraspecific antivenom in a rabbit model.
8. To study the proteomic profile of *H. hypnale* venom.
9. To isolate and characterize the major procoagulant enzyme from *H. hypnale* venom.

CHAPTER 2

Literature Review

2.1 Snakes: evolution and taxonomy

2.1.1 Evolution of the Serpentes

The amniotes advanced their Labrynthodonts predecessors (specialized, toothed ancient amphibians) by developing terrestrially-adapted eggs, i.e. amniotic or shelled eggs which could be laid on land, as typified by basal amniotes such as *Casineria* (~340 mya in the Mississippian epoch, Lower Carboniferous Geologic Period) (Laurin and Reisz, 1995; Monastersky, 1999; Paton *et al.*, 1999). Traditional classification recognizes Reptilia as a class of the amniotes, but this it has come under critique from cladistics as the class is indeed paraphyletic with two other classes (Mammalia and Aves) being given rise by the reptiles as defined. Based on phylogenetic studies, the clade Amniota was instead shown to have diversified into two main classes: the Synapsida (mammals/ reptilian-like stem-mammals and their extinct kinds) and the Sauropsida (true reptiles and other amniotes more closely related to reptiles than to mammals) (Gauthier *et al.*, 1988; Benton, 2004; van Tuinen and Hadley, 2004). The latter evolved into several clades along which included the extremely diverse Diapsida, which gave rise to the extinct lineages e.g. dinosaurs and pterosaurs; and the living crocodiles, tuatara, lizards, snakes, as well as birds (Aves) (Gauthier, 1994; Laurin and Gauthier, 1996).

One major obstacle in the studies of snake evolution is that the vast majority of snakes are small with typically fragile skeletons, making fossilization uncommon. Putative fossils, readily identified as snakes, dating as far back as 150 million years to the late Jurassic period have been discovered, though the traces were evanescent (Durand, 2004). Yet, fossils with lizard-like skeletal structures, representing some transitional forms have been found: there were no less than four separate genera, dating back to the

Cretaceous period, that were equipped with stubby, vestigial hind legs. Three of these, the *Eupodophis*, *Haasiophis* and *Pachyrhachis*, were discovered in prehistorical marine environment in the Middle East (Caldwell and Lee, 1997; Rieppel *et al.*, 2003; Houssaye *et al.*, 2011), consistent with the hypothesis that snakes once evolved from water-dwelling reptiles. However, *Najash*, a fossil from the Cretaceous of Argentina bore not only robust hind limbs but a sacrum that allowed the limbs to articulate with the backbone, was probably the most primitive snake yet known, and its anatomy suggests a terrestrial, burrowing origin for snakes (Apesteguía and Zaher, 2006). The prevailing view today is that snakes descended from burrowing terrestrial varanoid lizards, at least that the two share a common ancestor. Hence they evolved in parallel to the varanids and the helodrems, which are the New World venomous lizards and newts, to which they are still close (Chippaux, 2006). Through millions of years, and through several events of tectonic plates changes on Earth, snakes of different sizes and physical characters appeared and disappeared depending on the available environmental niches and how “fit” one was to cope with the force of Nature Selection. To date, the colubrids (of Colubridae), representing the “typical modern” snakes, with smaller body size but faster movement than the more primitive boids and pythons, make up over two-thirds of all living snake species (Greene, 1997; Vidal, 2002) .

An interesting (perhaps the most important) progress seen in the reptilian evolution was the emergence of venom-producing species. Reptilian venoms used to be thought as independently developed among the venomous lizards, newts and serpents – even so among the various seemingly polyphyletic families of venomous snakes as a result of convergent evolution 60-80 mya. Surprisingly, recent discoveries of venom toxins in snake subfamilies previously thought to lack them (Fry *et al.*, 2003a; Lumsden *et al.*, 2005; Pawlak *et al.*, 2006), and the evidence that nearly all non-venomous snakes

produce venoms to a certain extent (Fry *et al.*, 2003c, 2008; Fry, 2005), suggested a single, and thus far more ancient origin for venom in Serpentes than had ever been considered. Phylogenetic study by Vidal and Hedges (2005) dated the ancestry of venomous reptiles back at least to 200 mya to the Late Triassic/Early Jurassic, before/during the divergence among anguimorphs, iguanians and ‘advanced’ snakes, which formed a hypothetical venom clade named “Toxicofera”. Fry *et al.* (2006) and Vonk *et al.* (2008) subsequently showed that the evolutionary origin of reptilian venom proteins and their delivery systems may indeed have rooted deep in the phylogeny of squamata, across the families Caenophidia, Anguimorpha, and Iguania (also see section 2.2). These findings revolutionize the knowledge of evolution and systematics of venomous reptiles, which in turn provides a better understanding of venom distributions across snake families (Fry *et al.*, 2008). This greatly widens the research platforms in the field of toxinology: re-evaluation of the venomous status of snakes, comparison of the compositions and mechanisms of venom toxins, clinical study of envenomation and antivenom therapy, and drug discoveries from a larger venom reservoir full of pharmacologically active compounds previously under-explored.

2.1.2 Taxonomy of the snakes

Taxonomy and systematics of snakes are important in toxinology: they ensure replicability of research results, production of appropriate antivenoms, correct clinical diagnosis and the subsequent management. Snakes generally follow the scientific classification: Animalia: Chordata: Reptilia: Squamata: Serpentes (suborder). These are followed by two main groups, each representing an infraorder: the Scolecophidia (blindsnakes and threadsnakes, ca. 340 species) and the Alethinophidia (ecologically diverse, ca. 2640 species). Among the Alethinophidians is the superfamily Caenophidia (ca. 2470 species), considerably more advanced snakes in terms of predation behavior

by their widely use of venom and/or constriction. In the past one to two decades, several large bodies of phylogentic works resulted in extensive revisions of the systematics of snakes, especially those with medical and toxinological concerns. The advances in this aspect could not be possible without the use of molecular techniques in correlating the mitochondrial DNA, venom gland messenger RNA and venom chemistry that greatly complemented the ‘gaps’ in fossil records for visualizing the evolutionary interrelationships among the species. Recent molecular phylogenetic studies showed a near consensus of the evolutionary relationship in the Caenophidia, that the Caenophidians evolved from a single common ancestor into several (super)families where each represent an independent monophyletic lineage within the Caenophidia (Slowinski and Lawson, 2002; Fry *et al.*, 2003b; Lawson *et al.*, 2005; Vidal *et al.*, 2007). More interestingly, the ancestor was a venomous one with at least some parts of a venom delivery system; and this was later shown as homology in all venomous squamata (Toxicofera), dating the venom evolution to a much earlier time (Vidal and Hedges, 2005; Fry *et al.*, 2006). However, the translation of these phylogenetic data into a stable family-level taxonomy of venomous snakes has not been reached (Lawson *et al.*, 2005; Vidal *et al.*, 2007). A more restrictive approach adopted by Vidal *et al.* (2007) in the classification of venomous snakes (Caenophidia) is presented in Figure 2.1; while snake genera with medical or toxinological interests are shown in Table 2.1.

2.2 Advanced snakes: venom delivery system and toxin compositions

The venom delivery systems have evolved in several living groups of reptiles, classically the advanced front-fanged venomous snakes and helodermatid lizards (Kochva, 1978; Minton and Minton, 1980; Zug, 1993). Recent findings indicated that venom systems exist too in many rear-fanged snakes and non-helodermatid lizards, collectively grouped as the toxicofera (Fry *et al.*, 2006; Vonk *et al.*, 2008).

Among snakes, species within the Elapidae and Viperidae families, and, the sister genera *Atractaspis* and *Homoroselaps* within the Atractaspidine subfamily of the Lamprophiidae family, have elaborate, morphologically-specialized high-pressure front-fang venom systems, with a characteristic but different pattern for each group (Fry *et al.*, 2008). Snake venom glands are located posterior to the eye and extend in a line along the upper jaw, while ventrally the supralabial mucous glands extend along the entire length of the upper jaw. In all front-fanged snakes, venom is delivered through muscular compression of the glands that helps propel it along venom duct towards the fang (Figure 2.2a). Differing from non-venomous primitive constrictor snakes e.g. pythons whose teeth lack grooves (aglyphous), venomous snakes typically displayed the following three types of dentitions (Figure 2.3) (Gopalakrishnakone *et al.*, 1990; Mackessy, 2010a):

- ***Ophistoglyph***: rearward-grooved teeth, vernacularly termed rear-fanged due to the teeth position in the mouth. These snakes are mainly found in the family Colubridae.
- ***Proteroglyph***: forward-grooved teeth, positioned at the anterior end of the maxilla, generally short, unique to the elapids.

- ***Solenoglyph***: pipe-grooved teeth, anteriorly positioned and supported by a reduced “maxillary nub” at each side. These fangs can be as long as half the length of the head, foldable (against the upper palate), and rotatable in biting position. The dentition is unique to vipers and pit vipers, as well as *Atractaspis* (with slight difference). A typical solenoglyphous fang is shown in Figure 2.2b.

Although the solenoglyphous dentition displayed by viperids appears to be the most mechanically sophisticated and so generally thought to be the most derived, both a common origin and an independent evolution of the proteroglyph and solenoglyph systems have been inferred (Fry et al, 2009; Kardong, 1982). The caenophidian venom apparatus has indeed experienced extensive evolutionary tinkering with a wide range of independently changing variables (from biochemical variation to the variation in dentition and glandular morphology), thus giving rise to myriad toxins associated with diverse venom systems (Vidal, 2002; Fry *et al.*, 2008). The prominent front-fanged system appeared three times independently: once early with viperids, once within atractaspidines, and once within elapids, while the rear-fanged system, although evolutionarily appeared less efficient in injecting venoms, has maintained their toxin complexity suited for smaller and ‘simpler’ prey. Interestingly, further reduction in the size and complexity of venom system has been subsequently observed in species in which constriction evolved secondarily as the preferred method of predation, or when dietary preference switched from live prey to eggs or slugs/snails (Vidal & Hedges, 2009).

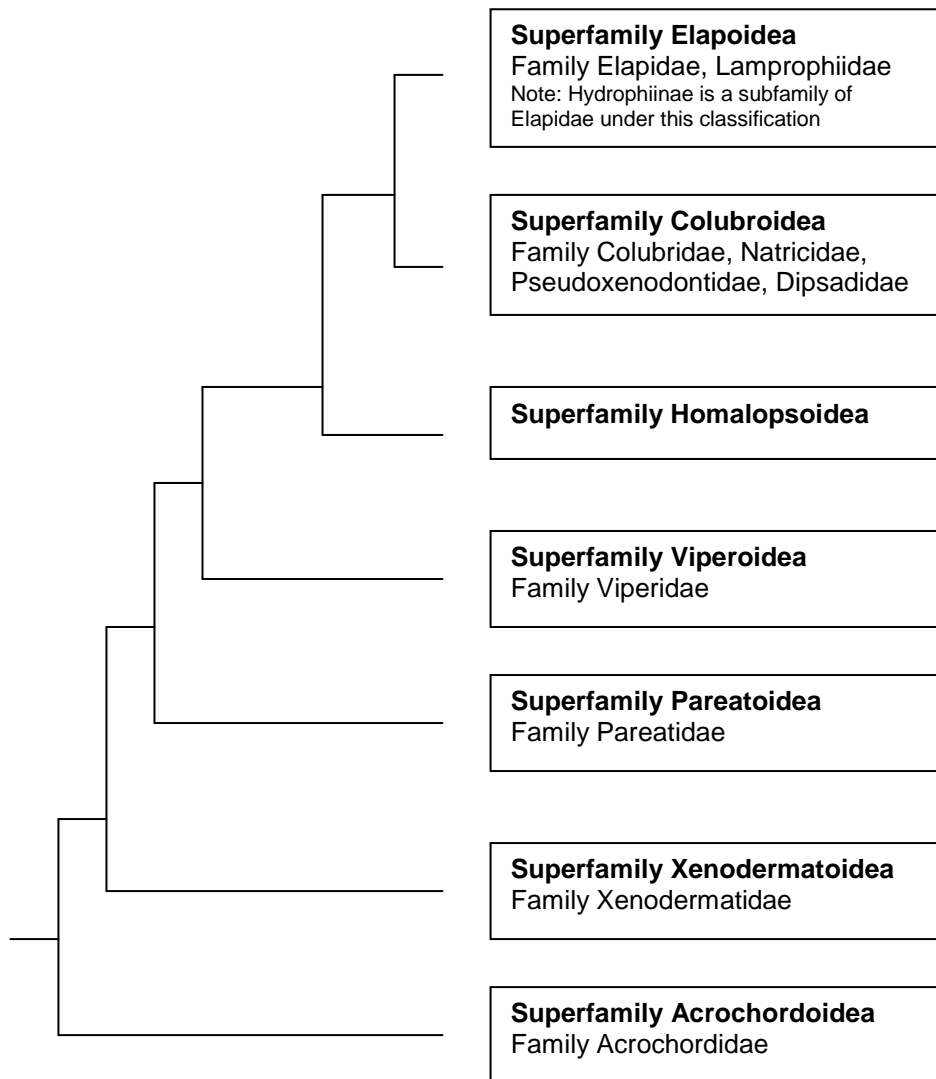


Figure 2.1 Simplified taxonomy and evolutionary higher-level relationships in the Caenophidia based on molecular phylogenetic study by Vidal *et al.* (2007).

Table 2.1 Family-level classifications of snake genera (with subfamily if indicated) of documented or potential medical and toxonological interest.

Family	Viperidae	Homalopsidae	Colubridae	Dipsadidae	Natricidae	Lamprophiidae	Elapidae
Subfamily:	Azemiophinae:	<i>Cerberus</i>	<i>Ahaetulla</i>	<i>Alsophis</i>	<i>Amphiesma</i>	Atractaspidinae:	Elapinae:
Genus	<i>Azemiceps</i>	<i>Enhydris</i>	<i>Boiga</i>	<i>Boiruna</i>	<i>Macropisthodon</i>	<i>Atractaspis</i>	<i>Aspidelaps</i>
		<i>Homalopsis</i>	<i>Coelognathus</i>	<i>Erythrolampr</i>	<i>Rhabdophis</i>	<i>Homoroselaps</i>	<i>Bungarus</i>
	Causinae:		<i>Leptophis</i>	<i>us Liophis</i>		<i>Macrelaps</i>	<i>Calliophis</i>
	<i>Causus</i>		<i>Oxybelis</i>	<i>Tachymenis</i>			<i>Dendroaspis</i>
			<i>Telescopus</i>	<i>Xenodon</i>			<i>Hemibungarus</i>
	Crotalinae:		<i>Trimorphodon</i>			Psammophiinae:	<i>Micrurus</i>
	<i>Agkistrodon</i>					<i>Malpolon</i>	<i>Naja</i>
	<i>Bothrops</i>					<i>Psammophis</i>	<i>Ophiophagus</i>
	<i>Calloselasma</i>						<i>Pseudohaje</i>
	<i>Crotalus</i>					Pseudoxyrhopiinae:	<i>Walterinnesia</i>
	<i>Cryptelytrops</i>					<i>Langaha</i>	
	<i>Deinagkistrodon</i>					<i>Madagascarophis</i>	
	<i>Gloydius</i>						Hydrophiinae:
	<i>Hypnale</i>						<i>Acanthophis</i>
	<i>Ovophis</i>						<i>Drysdalia</i>
	<i>Popeia</i>						<i>Enhydrina</i>
	<i>Sistrurus</i>						<i>Furina</i>
	<i>Trimeresurus</i>						<i>Hydrophis</i>
	<i>Tropidolaemus</i>						<i>Notechis</i>
							<i>Oxyranus</i>
	Viperinae:						<i>Pseudechis</i>
	<i>Bitis</i>						<i>Pseudonaja</i>
	<i>Cerastes</i>						<i>Toxicocalamus</i>
	<i>Daboia</i>						<i>Vermicella</i>
	<i>Echis</i>						
	<i>Macrovipera</i>						
	<i>Vipera</i>						

Simplified from Mackessy (2010a); based on the findings of widely distributed venoms in the Caenophidia (Fry *et al.*, 2003b; 2008), and the taxonomic revision proposed (Vidal *et al.*, 2007).

If the venom gland-fang complex has the analog of a syringe-and-needle instrument, venom is therefore the essential ‘dose’: following bite, it is injected and disseminated into the recipient’s biological system, in which it shall alter a wide range of physiological responses, often deleterious. Generally, venom is regarded as a complex secretion produced in a specialized gland, typically delivered via specialized systems that include the gland, specialized teeth/stings, and a suite of specific behaviors allowing the introduction of the venom into recipient tissues, usually by injecting (not ingested as in the case of poisoning) (Makessy, 2002; Vonk *et al.*, 2008).

The great diversity of snake venom toxins is due to their mode of evolution where diet plays the central role in the adaptive radiation of snakes (Daltry *et al.*, 1996). Predator-prey arms race (Heatwole and Poran, 1995) contributes to repeated gene duplication that creates redundancy and allows a gene copy to be selectively expressed in the venom gland, and subsequently undergoes neofunctionalization through positive selection and adaptive molecular evolution at accelerated rate (Kini and Chan, 1999; Kordis and Gubensek, 2000; Calvete *et al.*, 2007b). Gene duplication followed by functional-structural diversification as seen in such scenario is a typical example for molecular novelty. The occurrence of multiple isoforms within each major toxin family also evidences the emergence of paralogous groups of multigene families across taxonomic lineages where gene duplication events occurred prior to their divergence, suggesting an important role for balancing selection in maintaining high levels of functional variations in venom proteins within populations (Calvete *et al.*, 2009).

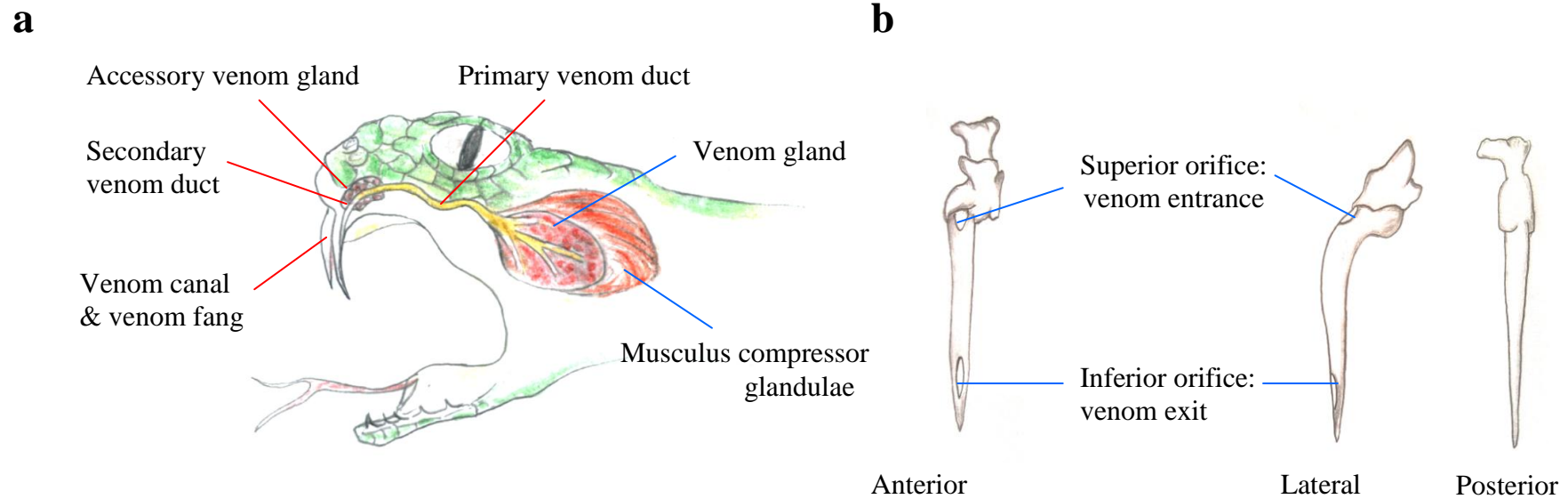


Figure 2.2 Schematic representation of (a) structure of the venom gland-fang complex in a typically forward-fanged snake; (b) orifice found on a typical pipe-grooved fang. Venom-delivery machinery with pipe-grooved fangs anteriorly positioned in the mouth is seen in Viperidae snakes (true vipers and pit vipers). From the venom gland the venom is channeled into the superior orifice and injected into recipient tissue through the inferior discharge orifice, which is strategically opened anteriorly and above the solid tip of the fang to avoid plugging by soft tissue in the case of a hollow tip opening, and to minimise expulsion of venom if the tip penetrates the body of a small prey.

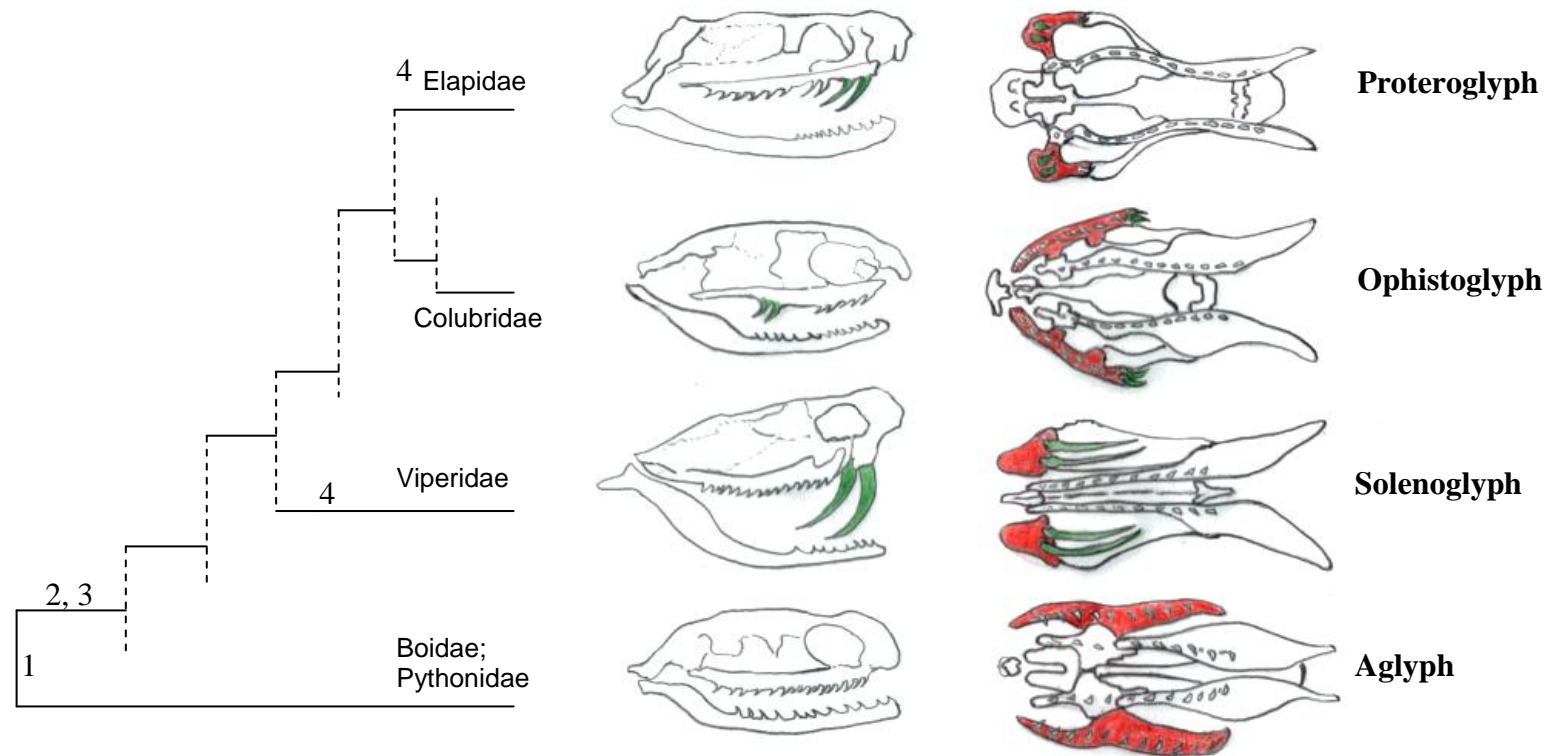


Figure 2.3 Types of fang in advanced snakes with their relative dental positions (fangs and maxillae colored green and red, respectively) mapped onto their phylogeny in brief. Numbers indicate important evolutionary changes leading from an unmodified maxillary dentition to the different fang types in advanced snakes: (1) continuous unspecialized dental lamina; (2) developmental uncoupling of posterior from anterior teeth (evolution of posterior dental lamina); (3) differentiation of the posterior teeth with the venom gland began; (4) loss of anterior dental lamina and development of front fangs (Vonk *et al.*, 2008). (Picture was reproduced by drawing with modification.)

The biochemical composition of venoms among snake species (or even within a species itself) is known to vary greatly, although in general, the dominance of major protein families in venoms often follows broad taxonomic trend and is hence partially predictable. For instance, low molecular weight three-finger toxins predominate within the elapid venoms, while the viperid venoms are richer in high molecular weight enzymes of which many are proteases (Mackessy, 2010a). The multiplicity of toxins exhibits synergistic interactions that primarily function to aid predation and digestion; although in some instances (as in human encounter), envenoming usually reflects a self-defense mechanism adapted by the snakes. The multiplicity of venom toxins and actions also give rise to complex clinical syndromes, but certain predominant pathology that can be anticipated based on the biting species. Table 2.2 lists some of the venomous snakes and their major venom toxins/enzymes that are medically or toxinologically important.

With the understanding of snake high-level phylogeny and venom distribution among the advanced snakes, it is not surprising that considerable amount of taxonomic changes took place at not only family level but at the genus and species levels too (Wüster *et al.*, 1997; 2008). Recent advances in venomous snake systematics address the importance of correct species identity, especially relevant to toxinologists and clinicians, in safeguarding the validity of their research results and therapeutic approach for envenomed patients. In parallel to this, venom and toxin studies advanced tremendously in the past two decades; for example, the fractionation technology of venoms has become more complex and detailed, while molecular studies (genomics, transcriptomics, proteomics) now provide in-depth understanding of the expression of various toxins (Chippaux, 2006; Calvete *et al.*; 2009, Jiang *et al.*, 2011). These achievements enable individual toxins to be studied beyond

Table 2.2 Some medically or toxinologically important snakes with their major venom components and general characteristics.

Family Genera	Important component	Molecular mass (kDa)	Biological activity or effect	References
Viperidae	L-amino acid oxidase	85-150	Cell damage, apoptosis	Tan (1998)
	Hyaluronidase	73	Dissemination of venom	Tu and Kudo (2001)
	Phospholipase A ₂ (Group II)	13-15	Cellular membrane damage, neurotoxicity, myonecrosis	Gopalakrishnakone et al., (1984, 1997); Kini (2003)
	Thrombin-like serine proteases	31-36	Hemostatic disturbance	Markland (1998a)
	C-type lectins	27-29	Anticoagulant and platelet modulator	Leduc and Bon (1998a)
	Metalloproteinases	20-40	Hemorrhage, myonecrosis	Fox and Serrano (2008)
	Phosphodiesterase	94-140	Hydrolysis of nucleic acids and nucleotides	Mackessy (1998)
	Alkaline phosphomonoesterase	90-110	Hydrolysis of phosphomonoesterase bonds	Rael (1998)
Elapidae	Three-finger toxins (cardiotoxin, α -neurotoxin, fasciculin etc)	6-9	Potent neuromuscular inhibitor, cardiac cytotoxicity	Kini (2002); Fry <i>et al.</i> (2003b); Nirthanan and Gwee (2004)
	Phospholipase A ₂	13-15	Neuromuscular toxicity, myotoxicity, lipid membrane damage	Kini (1997, 2003); Geh & Toh, (1978); Brook <i>et al.</i> (1987)
	Cysteine-rich secretory proteins/ Helveprins	21-29	Possibly inducing hypothermia and prey immobilization	Yamazaki and Morita (2004)
	Acetylcholinesterase	55-60	Neurotransmission disturbance	Anderson and Dufton (1998)
	L-amino acid oxidase	85-150	Cytotoxicity; anti-bacterial effect	Tan (1998); Lee <i>et al.</i> (2011)
	Hyaluronidase	73	Venom dissemination	Tu and Kudo (2001)
	Acetylcholinesterase	55-60	Neurotransmission disturbance	Hill and Mackessy (2000)
Colubridae	Three-finger toxins	6-9	Neuromuscular inhibitor	Fry <i>et al.</i> (2003b); Pawlak <i>et al.</i> (2006)
	Phospholipase A ₂	13-15	Myonecrosis, lipid membrane damage	Huang and Mackessy (2004)

**Enhydrina schistosa* is a sea snake of the subfamily Hydrophiinae

descriptive work for various diagnostic and therapeutic applications (Koh *et al.*, 2006), e.g. agents like ancrod, captopril, eptifibatide, tirofiban, reptilase etc.

2.3 Snake envenomation: epidemiology, management and challenges

2.3.1 Global epidemiology

Studies on the global epidemiology of snake envenoming have been fragmentary, and often posed a question of how representative local studies were of the wider epidemiological situation (Chippaux, 2008). Kasturiratne *et al.* (2008) estimated that at least 421,000 envenomings and 20,000 deaths occur worldwide from 5.5 million snakebites annually, although these figures may be as high as 1,841,000 envenomings and 94,000 deaths.

The vast majority of the estimated burden of snakebite is in South and Southeast Asia, sub-Saharan Africa, and Central and South America. Despite its high incidence of snakebite, mortality due to snake envenoming is relatively lower in Central and South America when compared to other high incidence regions, possibly due to better snakebite management systems, including the development of locally effective antivenoms in many Latin American countries. India, with its population of over a billion people, accounted for the highest estimated number of bites and deaths for a single country. Under-reporting is a known issue affecting the reliability of snakebite incidence estimation, and this is likely reflected in the lower estimates in sub-Saharan Africa (Kasturiratne *et al.*, 2008). One undisputable fact about snake envenoming is that it affects predominantly developing countries in the tropics/subtropics – endemic areas where snakebite victims often have poor access to healthcare centers, many of which are already suboptimal to start with. The problem has been aptly described as a “disease of

poverty”, and is known for its neglected status, where the solutions to such problem are possible only in the presence of a global partnership (Gutiérrez *et al.*, 2006; Harrison *et al.*, 2009).

2.3.2 Management

The management of snake envenomation entails first aid, diagnostics, ancillary treatment and most importantly, the definite antivenom therapy – the usefulness and safety of which depend greatly on correct diagnosis (WHO, 2005). Particularly challenging are issues related to the diagnosis of biting species and the efficacy as well as availability of antivenom. Snake diagnosis can be reliable if the species is directly identified by a herpetologist, but not all cases are presented with a captured or killed specimen at the hospital – in fact, it might be dangerous trying to capture the biting snake. Diagnosis therefore depends much on patients’ descriptions, or by monitoring the clinical syndrome to deduce the likely cause of offending snake (WHO, 2005). For instance, the development of neuromuscular paralysis is suggestive of elapid envenoming, while incoagulable blood and hemorrhagic syndrome is likely due to viperid bites in certain localities. However, a delay in diagnosis would inevitably result in late administration of appropriate antivenom when indicated. To date, there is no diagnostic kit available for rapid identification of the biting venomous snakes apart from the CSLTM snake venom detection kit used in Australia (White, 2001).

2.3.3 Antivenom for snake envenoming

In the late 19th century, Albert Calmette developed the ‘antivenomous serum’ (Calmette, 1896) which therapeutic principle remains valid to date. In current medical context, such therapeutic, termed antivenom, is the only etiological treatment for snake envenoming (Chippaux and Goyffon, 1998; WHO, 2010). Antivenoms are usually made

of polyclonal antibodies (immunoglobulin G, IgG) of heterologous origin, mainly horses and sheep which were immunized with the snake venom(s) (Theakston and Warrell, 1991; Gutiérrez *et al.*, 2003). Based on the molecular mass of the neutralizing molecules, generally three main types of antivenom products are available (Gutiérrez *et al.*, 2003): (i) whole IgG antivenom (~150 kDa); (ii) F(ab')₂ antivenom (~100 kDa); (iii) Fab antivenoms (~50 kDa). Antivenom exerts its *in vivo* pharmacological action by forming immunocomplexes with the venom toxins, which will be subsequently eliminated (by phagocytosis or renal route), hence neutralizing the deleterious effects of the toxins (Gutiérrez *et al.*, 2003; 2007). Due to its heterologous origin, patients given antivenom are at risk of developing hypersensitive response, which may manifest as early (anaphylaxis) or late (serum sickness) reactions (Malasit, 1986). Various laboratory efforts have been advocated to improve the product purity and safety, as well as to minimize the allergenic properties of antivenoms, besides reducing infection risks. These include methods such as pasteurization, nanofiltration, affinity immunopurification and ion-exchange chromatography (Smith *et al.*, 1992; Grandgeorge *et al.*, 1996; Raweerith and Ratanabanangkoon, 2003; Burnouf *et al.*, 2004; Khomvilai, 2008).

2.3.4 Challenges in the management of snake envenoming

Gutiérrez *et al.* (2006) highlighted various scientific, technological and political tasks needed in the form of global collaboration to improve snake envenoming prevention and treatment. The efficacy, quality and distribution of antivenoms are particularly of concern. The coverage and effectiveness of antivenoms depend greatly on the knowledge of venom properties and toxin variations, and this aspect has been frequently addressed and looked into by fundamental research. However, at the level of commercial production and distribution of antivenoms, there is still a serious lack of

antivenoms in many remote areas due to market failure (Chippaux, 1998, 2008; Theakston and Warrell, 2000). Suggestions that have been made to address this deficiency include technology transfer to facilitate local antivenom production, public-private partnership to make antivenom accessible at reasonable price, and better information on the global burden of snakebite so the antivenom manufacturers would be able to better regulate their production, while medical authorities could effectively allocate antivenoms to where they are most useful and needed (Gutiérrez *et al.*, 2006; Chippaux, 2008). In 2008, the Global Snakebite Initiative (GSI) was founded in collaboration with the International Toxinology Society and works toward the development of an integrated approach to overcome the long-standing antivenom crisis (Williams *et al.*, 2010). Mechanisms proposed include international collaboration on evaluating existing antivenoms, and the application of venom proteomics in the production of polyvalent antivenoms with improved potency and quality (Williams *et al.*, 2011).

2.4 Toxinology of snake venom: some laboratory aspects

2.4.1 Characterizing the toxic effects

In envenoming, the clinical presentation of a victim represents a complex syndrome resulted from the body responses to the pharmacological actions of different components in the snake venom. Venom toxic effects have often been conveniently classified as neurotoxic, hemotoxic, cardiotoxic, nephrotoxic, myotoxic and so on based on the predominant clinical effect of a particular venom. Such targeted organ system-based descriptions at times receive critique that the classification seemingly oversimplifies the complexity of venom effects and does not represent the interaction of various toxins on tissues from different organs and the resultant complex systemic

response. Nevertheless, the descriptions have been commonly used especially at clinical setting, as they generally denote the prominent clinical syndrome observed and anticipated. This simplified classification of the venom effects probably has more of a practical value in planning for monitoring and management; for example, the preparation of intubation facility, blood products for transfusion, dialysis facility and so on, in anticipation of the likely pathological outcome.

On the other hand, laboratory characterizations of venom toxicities cannot be oversimplified and it necessitates multiple disciplinary approaches, involving both *in vitro* and *in vivo* methods. Venom toxinological studies investigate the fundamental toxic effects of a venom for better insight into its pathophysiology, and aim to establish parameters that can be useful for future references, especially in neutralization study. Table 2.3 summarizes some essential aspects in toxinological profiling of snake venoms, highlighting some commonly used experimental methods and outcomes.

2.4.2. Separation of snake venom components

Separation (and identification) of venom components is achieved by two commonly used methods: electrophoresis and liquid chromatography (Chippaux *et al.*, 1991). In the past one to two decades, optimization and modification with sophisticated technologies, such as the combinational use of isoelectric focusing, 1- or 2-dimensional electrophoresis, high performance liquid chromatograph, and mass spectrophotometry enable most venom toxins to be fractionated to the purest and identified even at nanogram level (Li *et al.*, 2004; Calvete *et al.*, 2009). Detailed characterizations of a purified individual component are necessary for elucidating its mechanism of action, developing antidote for inhibition and neutralization, as well as therapeutic application in drug discovery.

Table 2.3 Important aspects for characterizations in toxinological profiling of snake venoms.

Biological and toxic activities	Example	Method and observation	References
Enzymatic activities	Phospholipases A ₂ Thrombin-like enzyme Proteolytic enzyme	<i>In vitro</i> ; phospholipid degradation <i>In vitro</i> ; fibrinogen clotting <i>In vitro</i> : caseinolysis	Tan and Arunmozhiarasi, (1989) Collins and Jones (1972) Tan <i>et al.</i> (1986)
Hemorrhagic activity	Hemorrhagin	<i>In vivo</i> : intradermal bleeding	Tan and Saifuddin (1990)
Necrotic activity	Viperid venom	<i>In vivo</i> : dermal necrosis	Theakston and Reid (1983)
Lethality	A compound effect, may be predominated by one or more principal toxins	<i>In vivo</i> : median lethal dose (LD ₅₀) is expressed from a dose-response curve	Theakston and Reid (1983)
Cytotoxicity	Cytotoxic components in snake venom	<i>In vitro</i> ; cell death and apoptosis on cell culture <i>In vivo</i> ; reduced tumor size in mice	Damico <i>et al.</i> (2007) Gebirim <i>et al.</i> (2009)
Neurotoxicity	Neurotoxic components especially in elapids; neurotoxins	<i>In vitro</i> ; isolated chick biventer cervicis or phrenic nerve-diaphragm preparation showing neuromuscular blockade <i>In vivo</i> ; whole animal study under anesthesia showing respiratory muscle paralysis	Harvey <i>et al.</i> (1994) Geh (1993)
Myotoxicity	Myotoxins, many are phospholipases A ₂ and cytotoxins	<i>In vitro</i> : direct damage on chick biventer cervicis; skeletal muscle cell death on cell culture <i>In vivo</i> : myonecrosis in mice	Harvey <i>et al.</i> (1994) Geh and Toh (1978) Núñez <i>et al.</i> (2001)
Cardiotoxicity / vascular effect	Cardiotoxic components, many are cytotoxins from elapid venoms	<i>In vitro</i> : isolated rat atria or endothelium-intact aorta showing decreased contractility <i>In vivo</i> : whole animal study showing decreased cardiac output and blood pressure; might be accompanied with electrocardiogram changes	Lee <i>et al.</i> (1967); Bell <i>et al.</i> (1998) Geh (1993); Chanhom <i>et al.</i> (2010)
Nephrotoxicity	Secondary to hemodynamic changes (indirect effect), or caused by direct cytotoxic effects of the venom	<i>In vitro</i> : isolated perfused kidney showed reduced glomerular filtration rate and cell injury <i>In vivo</i> : whole animal showed altered renal hemodynamics and blood/urine biochemistry abnormality	de Castro <i>et al.</i> (2004) Chanhom <i>et al.</i> (2010)
Hematotoxicity (hemostatic disturbance)	Procoagulant enzymes (purpurase) Fibrinolytic enzymes (ussurenase) Platelet aggregation inhibitor (rhodocetin)	<i>In vitro</i> : formation of friable fibrin clots, fibrinogenolytic assay <i>In vitro</i> : hydrolysis of fibrin clot <i>In vitro</i> : inhibition of platelet aggregation	Tan (2011) Sun <i>et al.</i> (2006) Wang <i>et al.</i> (1999a, b)

It is noteworthy that most of the separation techniques employ successive use of various liquid chromatographies, e.g. gel filtration, ion exchange and/or affinity columns, to ensure a pure entity is recovered with minimal loss of activity (Ponnudurai *et al.*, 1994; Peichoto *et al.*, 2009; Tan *et al.*, 2012). Reverse-phase chromatography is a useful separating tool for venom proteins, but like electrophoretic separation, it is mostly reserved for physicochemical studies as the experimental condition may be harsh enough to render loss of biological activity of the components.

More recently, with the advancement of venom gland genetic database and peptide mapping technology, studies of the identities of various proteins expressed in different snake venoms become feasible and reliable (Li *et al.*, 2004; Serrano *et al.*, 2005). This strategy has been termed venomics, based on proteomic approach in which the global expression of proteins in a mixture is studied (Calvete *et al.*, 2009). This method is possible for identifying proteins from venoms of different species, due to the presence of high consensus regions within the peptides, consistent with gene duplication over evolutionary time that resulted in a structurally conserved protein family. Using sophisticated instruments like mass spectrometry coupled with bioinformatic technology, it is now possible to unravel snake venom composition at the molecular level (see Section 2.7.1-2.7.2). Following the development, antivenomics – a proteomic approach in studying antivenom neutralization of particular important toxins in venom was reported, with implication in the design and production of toxin-specific antivenom, believed to have improved effectiveness and lesser allergenic properties (Calvete *et al.*, 2009; Gutiérrez *et al.*, 2009).

2.4.3 Neutralization of venom effects by antivenom

Studies of neutralization of snake venoms are important preclinical assessment for validating the potential clinical use of an antivenom. Neutralization studies require prior understanding of the venom's toxic effects that should be abolished or attenuated by the treatment of antivenom. All clinically relevant toxic effects (neuromuscular depressant, hemorrhagic, necrotic, procoagulant, or nephrotoxic activities) are important aspects to be assessed in neutralization, although lethality is the single most vital activity that must be effectively neutralized (Bogarín *et al.*, 2000). Both *in vitro* and *in vivo* neutralization methods are commonly adopted: *in vitro* neutralization is achieved by preincubation of venom and antivenom followed by testing the incubate on animals, substrates, or isolated tissue preparation; while *in vivo* neutralization involves administering venom and antivenom independently into an animal (Lomonte *et al.*, 2009). It is of upmost importance to ensure that the experiment protocols are strictly controlled and standardized, as the results are indicative of therapeutic potentials for clinical application. Methodology details such as challenge doses, routes of administration, animal strains, experiment end points, and definitions of 'effective doses' should all be explicitly stated. Some clinically relevant snake venom toxic effects along with the methods to study their neutralization by antivenom are shown in Table 2.4.

Table 2.4 Some medically important venom effects and neutralization by antivenom studies.

Toxic effect	Challenge dose	Methodology used	Effectiveness measurement	Venom	Reference
Lethality	3-5 LD ₅₀	Preincubation with antivenom for 30 min followed by i.v. or i.p. injection	ED ₅₀	<i>Bothrops</i> sp.; <i>Naja</i> sp., <i>Echis ocellatus</i>	Bogarín <i>et al.</i> (2000); Ramos-Cerrillo <i>et al.</i> (2008)
Lethality	Predetermined lethal doses	i.p. or i.v. injection of venom followed by i.v. or i.m. injection of antivenom independently	Survival ratio	<i>Bothrops asper</i> ; <i>Micrurus nigrocinctus</i>	Léon <i>et al.</i> , (1999, 2001); Chaves <i>et al.</i> (2003)
Procoagulant	2 MCD	Preincubatio with antivenom for 30 min followed by plasma clotting test	ED	<i>Bothrops</i> sp.	Bogarín <i>et al.</i> (2000)
Hemorrhagic	3.5 MHD	Preincubation with antivenom for 30 min followed by i.d. injection	ED ₅₀	<i>Echis ocellatus</i>	Ramos-Cerrillo <i>et al.</i> (2008)
Necrotic	2.5 MND	Preincubatio with antivenom for 30 min followed by i.d. injection	ED ₅₀	<i>Echis ocellatus</i>	Ramos-Cerrillo <i>et al.</i> (2008)
<i>In vivo</i> defibrinogenation	3.2. MDD	Preincubation with antivenom for 30 min followed by i.p. injection	Neutralizing dose	<i>Echis ocellatus</i>	Ramos-Cerrillo <i>et al.</i> (2008)
Neurotoxicity	1 µg/ml	Antivenom added at time when venom produced 90% inhibition on indirect twitches in an isolated chick biventer cervicis neuromuscular preparation	Magnitude (%) of reversal of muscle twitch blockade	<i>Enhydrina schistosa</i> , <i>Lapemis curtus</i> , <i>Laticauda colubrina</i> , <i>Aipysurus</i> sp.	Chetty <i>et al.</i> (2004)
Myotoxicity	Predetermined myonecrotic dose	Venom injected i.m. in mice, followed by i.v. antivenom at various time points	Plasma creatinine kinase level as a quantitative index of myonecrosis	<i>Micrurus nigrocinctus</i>	Léon <i>et al.</i> (1999)
Nephrotoxicity	250 µg/ml	Antivenom added at various time points following venom onto isolated renal proximal tubule preparation	Level of lactate dehydrogenase (LDH) release indicated extent of cellular injury and reversal.	<i>Bothrops jararaca</i>	de Castro <i>et al.</i> (2004)

LD₅₀: median lethal dose; MCD: minimum coagulant dose; MHD: minimum hemorrhagic dose; MND: minimum necrotic dose; ED: effective dose; ED₅₀: median effective dose.

The efficacy of an antivenom can be quantified and expressed, usually as median effective dose (ED_{50}), determined from its dose-response curve or by statistical means. Variations of efficacy determination however are not uncommon, notably due to differences in venom challenge dose and routes of administration (apart from inevitable batch variations of venom and antivenom). It is known that neutralization studies with venom and antivenom do not strictly follow the “law of multiple proportions”, and the absorption rate of lethal toxins into blood determines the onset of systemic effects, which are usually the fatal ones (Bogarín *et al.*, 2000). Generally, the efficacy of an antivenom to neutralize the lethal effect of a venom is lower when a higher challenge dose of the venom is given intravenously. In order to standardize the comparison of neutralizing efficacy on lethality and to avoid ambiguity using ED_{50} alone, the parameter ‘potency’, P , has been adopted by some researchers as it is independent of the number of LD_{50} used in challenge (Araujo *et al.*; 2008; Morais *et al.*, 2010).

Binding of antivenom (paratopes) to venom toxin (epitopes), which strength is described by affinity, is the most fundamental action of the antivenom in neutralization of venom toxic effects. Generally, the molecular regions of the toxins involved in the interaction with their targets are blocked, precluding their toxic action. Such blockade may be achieved by direct or indirect mechanisms: (a) binding of the antivenom to epitopes which are the ‘active site’ of the toxin, (b) antivenom binds to epitopes in the vicinity of the toxin’s ‘active site’, providing steric hindrance over it, and (c) antivenom recognizes an epitope distant from the toxic site, subsequently induces conformational changes in the toxin molecule (Gutiérrez *et al.*, 2003). The recognition and binding of antivenom-toxin are species-specific, although in many instances an antivenom can cross-react with heterologous venom toxins (not included in immunization scheme for antivenom production), presumably due to the presence of common antigenic properties

between the different venoms. This may result in cross-neutralization, a well-known phenomenon in antivenom studies: where the antibody raised against a specific venom is capable of neutralizing the toxic effects of a heterologous one (Otero *et al.*, 1996; Seddik *et al.*, 2002; Rodríguez *et al.*, 2012). Typically, these species are phylogenetically closely related with similar ecological niche, implying well conserved structure-function properties of their toxins. Cross-neutralization has important implications as existing antivenoms can be of therapeutic use for envenoming cases by heterologous snakes even in another country. This is beneficial in terms of antivenom utilization, as the manufacturing of antivenom costs highly and is associated with many marketing obstacles (Krifi *et al.*, 1999; Chippaux, 2008), hence it is practically quite impossible for many under-developed countries to embark on such industry all on their own.

2.5 Immunological approaches in studying snake venoms

2.5.1 Snake venom antibody production for therapeutic purpose

During the late 19th century serum therapy was pioneered in treating microbial infections (diphtheria, tetanus) as well as “snakebite poisoning”. Over a period of more than a century, tremendous progress in the science of immunology has enabled the treatment of many microbial infections to “evolve” into a stage of preventive medicine, i.e. vaccination, which principle, in brief, is the generation of protective immunity iatrogenically achieved by repeatedly challenging the host with detoxified/inactivated toxin(s) or toxin derivatives. Snake venoms, comprised of various substances, have however not lent themselves to human vaccination to date even though interesting attempts existed, either by self-experimentation (Kagen & Muthiah, 2004) or clinical trial (Sawai *et al.*, 1969). A recent case of naturally passive acquisition of immunity was

reported in an elderly snake handler previously bitten by an Australian death adder (*Acanthopis* sp.), evidenced by high circulating IgG titer that rendered his blood venom level low in subsequent *Acanthopis* bite; however, the condition did not prevent local envenoming effect (myonecrosis) (Isbister *et al.*, 2010). Although theoretically feasible, human vaccination against snake envenomation, with its questionable efficacy, safety and ethics concerns, has never been validated for any realistic clinical application. Anti-snake “serum therapy” thus remains amongst the few centennial medications still in use today. While the mode of sourcing antibody (from animals) has not changed much, the methods of purification and utilization of the antibody have improved profoundly thereon, leading to the abandonment of the term “serum therapy” in favor of a more concise terminology: “immunotherapy”, or “antivenom therapy” (Chippaux, 2006).

Generally, the immunization protocol depends on the toxicity and the immunogenicity of venom, the species of animal to be immunized and the quality of immunological response of the hyperimmunized animal. Most commonly used animals are mammals of large body size such as horses (Theakston and Warrell, 1991; Gutiérrez *et al.*, 2003). Adjuvants, e.g. Freund's adjuvant, are added to amplify the immunogenicity of the venom, and repeated injections (10 to 15) spread over a period of three to fifteen months may be needed for a satisfactory antibody production in the animals (Chippaux, 2006). The antibody titers are monitored, usually by indirect ELISA, to determine if the antibody production has reached an optimal level for harvest. The choice of immunogen as one venom (monovalent) or several in the form of a mixture (polyvalent) depends on many considerations: types of venomous snakes and frequency of bites in the region, efficacy of neutralization, adverse effects and so on. Two questions concerning the benefits of monovalent over polyvalent antivenoms are whether it is more efficacious and whether it is less allergenic – based on the belief that less amount of antivenom

protein is administered than with the use of a polyvalent one. However, in reality there is often an observable higher neutralizing potency of polyvalent antivenom than that of monovalent antivenom. This could be explained by enhanced immunogenicity exerted by different venoms in a mixture for immunization, or due to cross-neutralization conferred by synergistic antibodies in the polyvalent form (Chippaux, 2006). This may in turn imply that less amount of polyvalent antivenom is required in treatment. Consequently, patients will have less exposure to heterologous antivenom proteins, and also a lower treatment cost incurred.

In many envenoming cases, identification of the biting snake based on patient's description may not be reliable; hence the choice of antivenom is usually empirically made according to patient's initial clinical signs and symptoms. In cases where diagnosis is ambiguous, a polyvalent antivenom, relevant to the local condition, seems to be the most appropriate choice of therapy. Besides, the manufacturing of polyvalent antivenom may effectively reduce the cost of high animal maintenance by minimizing the number of animals used and veterinarian care and hence relieving the burden of high production cost.

2.5.2 Immunodiagnostic assay for species identification

Diagnosis of biting snake species is clinically crucial as it guides towards the appropriate mode of monitoring or investigation and the choice of antivenom (WHO, 2005). Accurate identification of the species also has an important implication in epidemiology study, which will lend itself meaningless if the species documented are subjected to queries. In addition, species identification plays a vital role in forensic study (unexplained deaths and suicides suspicious of envenoming) (Selvanayagam *et al.*,

1999), as well as in legal cases concerning illegal trafficking of wildlife and venoms (Singh *et al.*, 2012).

The most reliable species identification would probably be seen in an ideal scenario where the victim brings along the biting snake, live or dead, to be identified by a herpetologist well versed in the taxonomy of local snakes, at the hospital – this however is not usually possible. In fact, misidentification is not uncommon even with the real snake being brought to the hospital (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008). From time to time, various techniques for venom detection and identification of snake species have been reported, including immunological assays and DNA-based molecular approaches (Theakson, 1983; Ho *et al.*, 1986a; Selvanayagam and Gopalakrishnakone, 1999; Dong *et al.*, 2003; Pook & McEwing, 2005). Enzyme-linked immunosorbent assay (ELISA), typically the double-sandwich or antibody-capture type, is likely the most widely studied diagnostic approach compared to polymerase chain reaction (PCR)-related technology which is much more technically demanding and cost-inhibitive.

Double-sandwich ELISA employs the principle of *ex vivo* antibody-antigen binding on a specially treated polystyrene surface, which interaction can be detected by a signaling enzyme-substrate reaction, e.g. horse-radish peroxidase activity (Figure 2.4). The antibody derived from the blood of an animal immunized with the venom or toxin of a particular species, has to be optimally purified to reduce non-specific binding that could lead to erroneous result interpretation. The purification can be achieved by affinity chromatography using Protein A or Protein G columns, depending on the source of animal plasma. Due to its sensitivity, the double-sandwich ELISA is also a commonly used method in determining blood venom levels in subjects following envenomation, both in clinical and laboratory studies (Ho *et al.*, 1990; Ariaratnam *et al.*, 1999;

Selvanayagam and Gopalakrishnakone, 1999). A limitation associated with double-sandwich ELISA diagnostic method is cross-reactivity that could possibly reduce its specificity. This usually affects venoms of closely-related snakes especially of the same family and locality, due to the presence of appreciable amount of common antigens in their venoms. Le *et al.* (2003) demonstrated the reduction of such cross-reactivities by developing a species-specific ELISA kit using purified toxins specific to venoms of different species, and the tool has now been applied in a national clinical trial. However, in many countries, research for snakebite immunodiagnostic kit remains an academic exercise, considering the various socioeconomical constraint faced by an under-developed country, with limited market demand and poor buying power that are unlikely to attract commercial investors. Currently, snake venom detection kit (immunodiagnostic method) is routinely used in Australia for its endemic venomous species (death adder, tiger snake, brown snake, black snake, and taipan) (White, 2001). Nonetheless, research effort in improving snakebite diagnostics should continue along with the call for global collaboration in addressing various challenges faced by the developing countries.

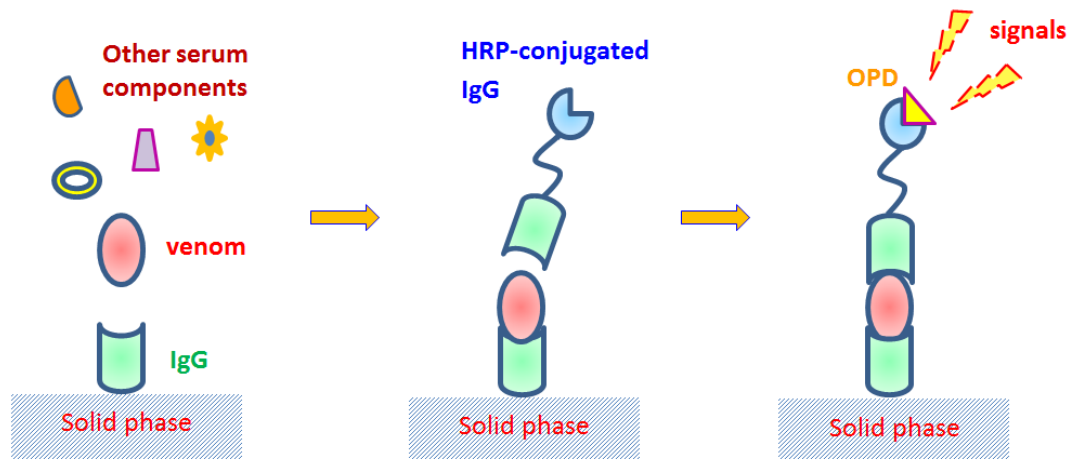


Figure 2.4 Fundamental principles of a double-sandwich ELISA (with horseradish peroxidase enzyme) in the detection of venom antigens. The coated IgG binds specifically to the venom proteins, while the other non-binders (serum components) will be eliminated through washing. With multiple epitopes, the venom protein antigens will be then bound by the secondary IgG which is HRP-conjugated. Addition of substrate (OPD) leads to HRP enzymatic reaction signaled as light absorbance.

IgG: polyclonal immunoglobulin G produced from immunization with a specific venom

Solid phase: polystyrene ELISA microtiter plate

HRP: horseradish peroxidase

OPD: o-phenylenediamine (substrate for horseradish peroxidase)

2.6 Pharmacokinetics of snake venoms

2.6.1 General approach in studying venom pharmacokinetics

Pharmacokinetics in classical sense refers to the study of a series of events a substance (usually a drug) undergoes when it is applied externally into a living organism. The processes involved are absorption, distribution, metabolism and excretion, with the latter two often collectively referred as elimination. The concept of pharmacokinetic model has been applied in the study of snake venoms, although literature on snake venom pharmacokinetics is apparently scarce compared with that on general biochemical characterizations of venoms. Venom pharmacokinetic studies were usually conducted on small laboratory animals like mice, rats and rabbits, making interspecies comparison and correlation of the data to human somewhat complicated, as pharmacokinetics depends greatly on various physiologic properties, which in essence are related to the organ mass size and body surface area of the animal. Added to this complex picture is the venom's nature as a mixture of various proteins, for which the 'pharmacokinetics' in strict sense needs to be interpreted carefully. In spite of these limitations, venom pharmacokinetic studies are essential in view of the scarcity of its database and the useful information that can be derived for improved understanding of the evolution of clinical syndrome and treatment protocols.

Venom pharmacokinetic experiments focus on the changes of venom concentrations in blood over a time course. Data from such studies provide information on the absorption, distribution and elimination of venom in a biological system, although, these data must be noted to be representative of various components in the venom as a whole. A purist approach in studying venom pharmacokinetics may involve studies on various toxins individually isolated from the whole venom, e.g. alpha-neurotoxins and thrombin-like

enzyme (Ismail *et al.*, 1996; Zhao *et al.*, 2001). However, the effects (and presumably the kinetic behaviors) of the venom are likely influenced by the interactions of various components in its mixture state, and hence may not optimally represent the profile of actual envenoming. Some independent variables that are important when designing venom pharmacokinetic experiments include the route of administration, and the time points, and duration of blood sampling. Intramuscular or subcutaneous injection of venom is certainly more representative of a natural bite, but may generate a more complex picture where certain parameters differ greatly from that of intravenous route. Sampling times are important especially in the beginning phase where distribution takes place as it provides crucial information for compartmental analysis.

Some of the aspects commonly discussed in the study of venom pharmacokinetics are listed below with their implications in the study of envenoming:

- (a) Compartmental model: This is a mathematical model without truly defining any anatomical space. Usually a 2-compartment model is described, with one compartment representing the peripheral tissues into which the venoms deposit, and the other compartment representing the blood and highly perfused organs where venoms circulate freely. A 3-compartment analysis is possible with certain toxins penetrating much further into deep tissues which form the third compartment. Equilibrium always exists among these compartments where the venom toxins transfer accordingly.
- (b) Distribution: In a 2-compartment model, this refers to the reversible movement of venom toxins to penetrate and bind onto peripheral tissues after they are introduced into the blood (through intravenous injection, or absorption from bite site). A larger

volume of distribution (V_d) signifies a higher affinity of the substance towards peripheral tissues than the circulating blood.

- (c) Elimination: This is the irreversible removal process of venom toxins following metabolism and/or excretion. The slower the elimination process, the longer the venom toxins remain in the system, and the likelihood of more profound toxic effects induced.
- (d) Clearance: It measures how fast venom toxins are removed from the system, usually expressed as unit volume of blood (being cleared of the toxins) per unit time. Total body clearance or systemic clearance is a summation of all clearance processes, whether by metabolism or excretion.
- (e) Half-life: Generally it refers to the time required for the venom concentration (in blood) to reduce to 50% of its initial level. It often differs between the phases of distribution and elimination, where the former is shorter (rapid distribution) than the latter (slow elimination). Conventionally, the plasma half-life refers to the elimination half-life.
- (f) Bioavailability: This is a measurement of the extent of venom toxins entering the systemic circulation through any route of administration. Bioavailability of a drug by i.v. route is 100%. However, intramuscular or subcutaneous injection would be more common in actual envenoming, and the venom bioavailability by these non-vascular routes may be lower than that of intravenous injection.
- (g) Area under the curve: This parameter can be used to estimate the amount of venom toxins in the blood over a time course. It is useful in the determination of venom bioavailability by non-vascular routes, and in comparing the extent of immunocomplexation in the presence of a neutralizing antivenom.

On the technical aspect, the venom dose chosen has to be within a quantifiable range of a sensitive assay; and the levels depicted should be meaningful to reflect an actual situation of envenoming. Preclinical assessment of venom pharmacokinetics on animal models usually employs the use of a radioisotope (e.g. iodine-131) coupled to the venom, or an enzyme (e.g. horse-radish peroxidase or alkaline phosphatase) conjugated to an antibody as in ELISA (Gutiérrez *et al.*, 2003). There are pros and cons in both experimental operations and the interpretation of data. Table 2.5 summarizes the major differences between these two methods of venom quantitation in pharmacokinetic study.

2.6.2 Changes of venom pharmacokinetics effected by antivenom and implications

The pharmacological action of antivenom is by principle the formation of immunocomplexes with venom components, rendering them inactive prior to elimination by phagocytosis. The therapeutic efficacy in actual bites, however, is greatly influenced by the time and route of administration as well as the posology/dosing regimen (Chippaux, 2006). Intravenous route of antivenom administration is recommended for fast action, in view of the fact that most venom components (with molecular weights lower than that of antivenom) distribute rapidly into tissues following absorption. The intravenous route is also preferred over intramuscular route as the latter has a much lower bioavailability. An effective antivenom injected intravenously can reduce the blood venom level drastically and the neutralizing effect persists as long as the amount of free antivenom is sufficient to bind to venom toxins that are slowly absorbed from injection site or re-emerge into the blood from peripheral tissues. However, a rebound venom level is often seen following the initial neutralization, presumably due to an enhanced venom transfer from tissues back into the

blood following a shift in the intercompartmental equilibrium following the law of mass diffusion (Chippaux, 2006). Other possible factors contribute to a resurgence in venom level following antivenom administration include slow absorption of venom from bite site while the venom is being eliminated slower than the antivenom. The phenomenon, often referred to as “venom redistribution” following immunotherapy, should be carefully addressed when designing the protocol for antivenom treatment (Gutiérrez *et al.*, 2003).

In addition, antivenom of different molecular types [whole IgG, Fab, F(ab')₂] have different pharmacokinetics especially related to elimination. Due to their larger molecular sizes (100-150 kDa), IgG and F(ab')₂ have a relatively longer elimination half-life as they are not readily removed by kidneys, and the affinity towards vascular compartment is high, i.e. less tissue-penetrating ability. Fab with a smaller molecular size (50 kDa) on the other hand, can be renally eliminated and hence has a shorter half-life. It however penetrates deeper tissues and is particularly useful in binding or displacing smaller toxins bound to receptors of the deep tissues. The diverse pharmacokinetics of antivenom and the complex absorption and distribution of venom components can potentially result in a mismatch of pharmacokinetic-pharmacodynamic relationship of the envenoming immunotherapy, affecting its clinical effectiveness (Seifert & Boyer, 2001). *In vivo* pharmacokinetic experiments are therefore important to complement neutralization tests by demonstrating an appropriate administration and dosing regimen for the optimization of the antivenom protocol.

Table 2.5 Major differences between the radioisotope and enzyme methods for measuring venom levels in pharmacokinetic studies.

	Radioisotope method	Enzyme immunoassay method
Technical preparation	<p>Coupling of radioisotope onto the venom components is needed prior to injection.</p> <p>Stability and half-life of radioisotope need to be closely monitored for validity of results.</p> <p>Special precautionary measures are needed against radiation exposure (e.g. I-131 decay gives off beta and gamma rays)</p>	<p>Antibodies (IgG) against the venom components need to be raised and purified prior to study.</p> <p>Enzyme conjugation to IgG is needed.</p> <p>Pure and enzyme-conjugated IgG generally can be stored (at -20 °C) without jeopardizing the stability and function for at least several months.</p> <p>Conjugated IgG serves as capturing antibody, while unconjugated IgG serves as coating antibody.</p>
Nature of inoculate	Venom components coupled to radioisotopes	Innate venom components without chemical conjugates
Measuring method	Radioactivity reading, ex vivo	Enzymatic activity detected as light absorbance, ex vivo
Influence on duration of study	Possible, radioisotope has a half-life to be taken into consideration (e.g. 8 days for I-131) when measuring the activity	Unlikely, as the venom exists in vivo in its innate form
Measuring target	All components which are coupled to the radioisotope including the degraded venom components	All antigenic components that are specific for the IgG binding
Detection of immunocomplexation during antivenom therapy	Unable, as the reading implies the totality of venom components (including both free venom components and immunocomplexes) before they were eliminated/excreted	Reliable, as the capturing IgG binds specifically to only the unoccupied antigenic sites of venom components not neutralized by the antivenom
Relevance to toxins and envenomation	Useful for measuring total venom level in the system regardless of the nature (toxicity and antigenicity) and conditions (free or antivenom-bound) of venom components	Generally more specific for measuring level of venom components that are antigenic (usually, the toxic proteins); and is able to indicate the extent of venom neutralization in the presence of antivenom
Practical clinical use	Not applicable	<p>Applicable in measuring the blood venom antigen level from human patients. Readings are representative of free un-neutralized venom components.</p> <p>Can be used to determine the presence and amount of venom distributed to various organs and tissues.</p>

Overall, an ideal antivenom should possess the following characteristics (Gutiérrez *et al.*, 2003):

- (a) High affinity towards the principal venom toxins
- (b) Similar rate and volume of distribution to that of the venom toxins
- (c) Slow elimination to ensure adequate neutralization of venom reaching the bloodstream later in the course of envenoming; and to accelerate venom redistribution (for removal) from tissue compartments.

2.7 Proteomic studies of snake venoms

2.7.1 Revealing the identities of venom proteins and the implications

Within the Class of Reptilia, toxicofera evolved and radiated for at least 200 million years and a distinct development accompanying these biological events is the production of venom of great diversity across various taxa (Fry *et al.*, 2006). The existence in the same venom of a diverse set of proteins (even of the same family) which differ from each other in their pharmacological effects well reflect an adaptive molecular evolution giving rise to molecular novelties (Calvete *et al.*, 2009). Characterizations of diverse bioactive components from venom (the majority are proteins and peptides) have a number of potential benefits for fundamental research, clinical studies, development of research and diagnostic tools, drug discovery, antivenom production strategies (Gutiérrez *et al.*, 2009); and even for correlation with taxonomy and evolutionary study of the species (Fry, 2005).

Traditionally, venom biodiversity study depends greatly on bioassay-guided fractionation, where fractions displaying the desired activity in a particular assay are

further characterized. The approach, however, does not address the many other potentially useful components as they do not possess the particular niche of activity being researched. An in-depth knowledge of the identity of proteins and peptides expressed in venom is therefore essential; not only for basic research purpose but also to better elucidate envenoming mechanisms as venom toxicity is often determined by the combination of toxins. Venom proteomic study, termed ‘venomics’ by some authors, aims to identify the various venom components (essentially proteins) of both high and low abundances. With a robust knowledge of the proteomics of a particular venom, the immunoreactivity of antivenoms against individual venom components can be then assessed by studying the extent of immunodepletion of particular toxins when the venom is incubated with an antivenom prior to proteomic analysis. This *in vitro* approach, termed ‘antivenomics’, is considered complementary to preclinical antivenom assessment on animal models, and it provides information for an optimization of antivenom formulation (Gutiérrez *et al.*, 2009).

2.7.2 Molecular approach in venom proteomic studies

The identity of a venomous protein is conventionally identified, following their separations, by antibody recognition or peptide N-terminal sequencing. Unfortunately, both these methods are inadequate for global analysis of proteins in venom. Recent advancement in proteomic field, with the availability of genomic sequences, powerful protein separating tools (various high performance liquid chromatographies, multi-dimensional electrophoresis), modern mass spectrometers with high sensitivity and high-throughput coupled with sophisticated computational methods, has made global analysis of venom proteins/peptides possible. This however depends greatly on the completeness of gene sequences of a particular protein, or at least those proteins closely homologous. As expected, snake venoms underwent molecular adaptations under

accelerated evolution, where gene duplications have greatly conserved many gene sequences of a protein family across various taxa, hence making the homology-orientated protein identification possible for closely related snake species. However, accelerated evolution also led to rapid amino acid divergence in which the peptide homology of some inter-species protein may appear low and deemed insignificant by automated search engine, although they indeed belong to a same protein family.

Proteomic study involves sophisticated analytical science that greatly depends on the use of mass spectrometry (MS). MS is a fundamental approach that can accurately determine the masses of intact protein or peptides for further deduction of the amino acid sequence (Liebler, 2002). Tandem mass spectrometry (MS/MS) analysis involves a series of events where precursor ions are selected in the initial stage of analysis, and subjected to fragmentation to produce product ions in the second stage of analysis. This is followed by the analysis of the product ions to obtain the amino acid sequence of a peptide: each experimental peptide mass is compared with the mass of the theoretical peptide produced by the digestion of the protein from a selected database (de Hoffmann, 1996; Marcotte, 2007). Where the sample is potentially a mixture of more than one protein, reverse-phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) will be useful, as used in several studies on venom proteins (Fry *et al.*, 2003c; Li *et al.*, 2004; Soogarun *et al.*, 2008).

The peptide sequences (internal or terminal) derived from MS/MS spectra were then matched to available protein databases for protein identification. Search engines that are commonly available include MASCOT, SEQUEST and BLAST, and search is done against protein database resources such as those available from the National Center for Biotechnology Information (NCBI), SwissProt and European Bioinformatics Institute (EBI). In the case where particular protein information is absent from the databases,

protein identification may be unsuccessful. It should be noted that venom proteins exhibit a wide spectrum of rapid amino acid sequence divergence, as expected under accelerated evolution (Ogawa *et al.*, 1995; Kordis and Gubensek, 1996; Ohno *et al.*, 1998), and hence the possibility of peptide ion spectra being unmatched to an existing database particularly for species which genes lack sequencing studies. Specifically, venom gland transcriptomics plays an important role in detailing the possible protein sequences in becoming “venom”. Unfortunately, transcriptomic data of such may not be always readily available in view of the cost, technology sophistication and concern on wildlife conservation. To overcome the limitation of matching venom ion spectra, a novel proteomic-bioinformatic approach has been described by Bringans *et al.* (2008) where purified peptides were analyzed by tandem mass spectrometry, *de novo*-sequenced and the homology of short sequences were matched against known peptides in a public protein database, using search engine such as BLAST (*Basic Local Alignment Search Tool*) that identifies regions of local similarity between sequences. The In the proteomic work by Bringans *et al.* (2008), some extents of statistical stringency were modified to enable the *de novo*-sequenced peptides matched to the existing database for protein identification, and subsequently the protein family is determined based on the putative function assigned.

Commonly employed modern methods of protein fractionation, digestion and sequencing in the studies of venom proteomes are shown in Table 2.6.

Table 2.6 Protein fractionation, enzymatic digestion and peptide sequencing methods in venom proteomic studies.

Fractionation of whole venom	Digestion of protein**	Sequencing of peptides	Reference
1-dimensional SDS-PAGE	In-gel tryptic digestion on excised gel bands	LC-MS/MS	Li <i>et al.</i> (2004)
2-dimensional electrophoresis*	In-gel tryptic digestion on excised gel spots	MS/MS for peptide fingerprinting N-terminal sequencing	Öhler <i>et al.</i> (2010)
(Not applicable)	Tryptic digestion of whole venom (shotgun analysis)	LC-MS/MS	Li <i>et al.</i> (2004)
Reverse-phase HPLC and 1-dimensional SDS-PAGE	In-gel tryptic digestion on excised gel bands	MS/MS for peptide fingerprinting; N-terminal sequencing	Calvete <i>et al.</i> (2007a) Angulo <i>et al.</i> , (2008) Lomonte <i>et al.</i> (2008) Sanz <i>et al.</i> , (2008) Fernández <i>et al.</i> , (2010)
Gel filtration HPLC and 1- or 2-dimensional electrophoresis	In-gel tryptic digestion of excised gel bands/spots	LC-MS/MS; or MS/MS for peptide fingerprinting	Li <i>et al.</i> (2004) Soogarun <i>et al.</i> (2008)

*2-dimensional electrophoresis is typically achieved by isoelectric focusing followed by SDS-PAGE based on molecular mass.

**digestion of protein is done enzymatically, usually with trypsin. Occasionally chymotrypsin can be used.

MS/MS: tandem mass spectrometry

LC-MS/MS: reverse-phase liquid chromatography coupled with tandem mass spectrometry

2.8 Snakebite in South Asia: with special reference to Sri Lanka

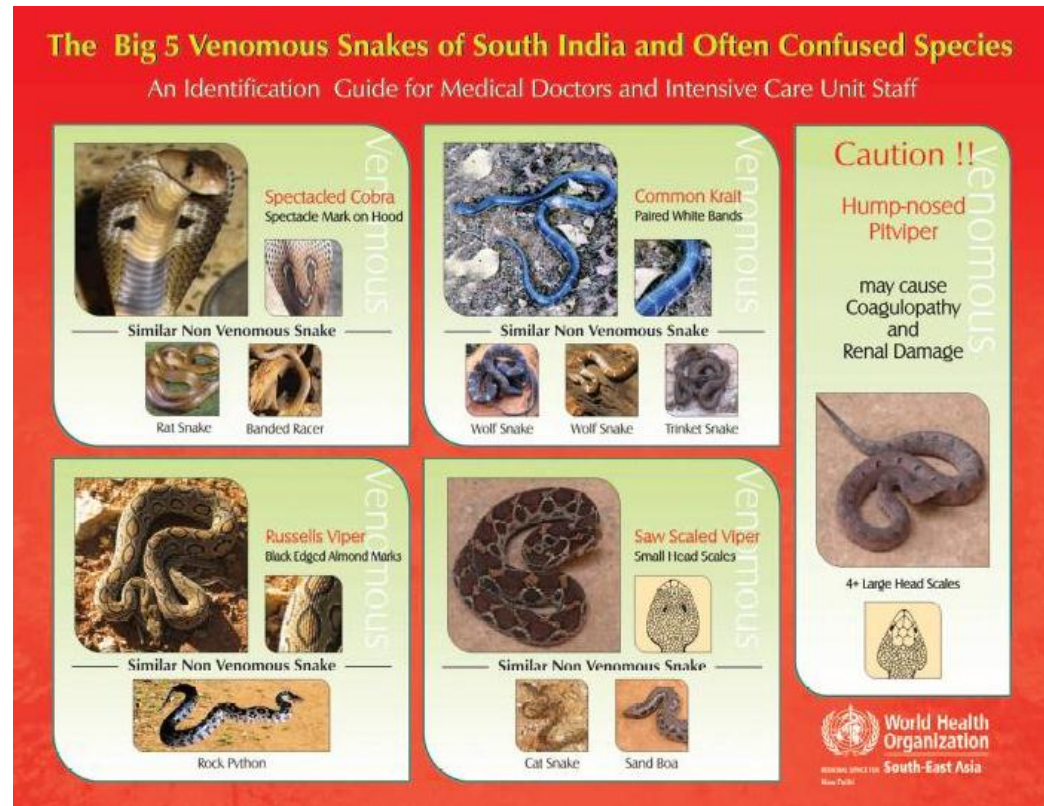
2.8.1 Biodiversity and medically important snakes

South to the Himalaya Range is one of the world's most bio-diverse regions, with India as one of the eighteen “megadiverse countries”, hosting two important biodiversity hotspots: the Himalayas and the Western Ghats. Sri Lanka, a large island country located south to India subcontinent, is closely associated with the latter for their striking similarities in geology, climate and evolutionary history, forming “a community of species” that validates both areas as one biogeographic unit (Myers *et al.*, 2000). The apparent biotic affinities within the Western Ghats–Sri Lanka biodiversity hotspot are the results of frequent migration during several extended periods of ocean-level lowstands, although the large number of endemics is likely credited to locally extensive radiation (Bossuyt *et al.*, 2004). Where venomous snakes are concerned, both areas are infested by a number of similar species. The “Big Four” (*Daboia russelii*, *Naja naja*, *Echis carinatus*, *Bungarus* sp.) used to represent the most highly venomous snakes in this region that cause most bites. Hump-nosed pit vipers, *Hypnale hypnale*, was recently added onto the list (Figure 1.3), as their bites were validated to be frequent, capable of causing severe complications and even death (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008). It presents two new challenges to the medical community: (1) there is no specific antivenom for treating *H. hypnale* envenoming; (2) revised clinical studies indeed showed that misidentification of hump-nosed pit viper was common, and the bite by this snake was estimated to be as frequent as that of *D. russelii* and *N. naja*. Other venomous snakes in the region include coral snakes, green tree pit vipers and sea snakes, which bites however appeared less frequently reported (Alirol *et al.*, 2010).

The similarity of biodiversity between the Western Ghats and Sri Lanka was indirectly reflected in some common features of snake envenomation affecting both areas. This implies that experience of snakebite management from one area may serve as a lesson to the other. Collaborative work especially pertaining to the improvement of antivenom coverage and efficacy would be of great mutual benefit to both sides. Currently, antivenoms used clinically in Sri Lanka are products of India against the Big Four, which have been proven clinically not effective against *H. hypnale* envenomation (Sellaheewa *et al.*, 1995; Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008).

Venomous snakes of medical importance in South India have been illustrated on educational poster (Figure 2.5a) and circulated by the WHO Southeast Asia Office, highlighting the status of “The Big 5 venomous snakes” that included the hump-nosed pit viper. In Sri Lanka, a guide jointly published by the Environmental Foundation and Wildlife Conservation Society classified the hump-nosed pit viper as a moderately venomous snake based on the venom toxic profile; however it emphasizes and agrees with the opinion of the Expert Committee on Snake Bite of the Sri Lanka Medical Association which re-classified the hump-nosed pit viper as a “highly venomous snake” status, due to its high prevalence and capability in causing severe dysfunction and death if not correctly treated (Figure 2.5b).

a



b



Figure 2.5 Educational publications regarding local venomous snakes in (a) South India; (b) Sri Lanka (inset is the enlarged portion for a statement found within the pamphlet).

Source: (a) retrieved from <http://www.flickr.com/groups/672105@N20/discuss/72157612167869325/>; (b) courtesy of Mr Dinal Selvator from the serpentarium at Faculty of Medicine, University of Colombo, Sri Lanka.

2.8.2 Hump-nosed pit viper (*Hypnale* species)

2.8.2.1 Zoology, distribution and systematics

Hypnale hypnale (hump-nosed pit viper) is by now considered as a highly venomous snake in Sri Lanka and southwestern coast of India (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008). It has been now listed as a snake of category I of medical importance by the World Health Organization (WHO, 2010), owing to its wide distribution and high prevalence of bites which cause significant morbidity and mortality in the region. Maduwage *et al.* (2009) reported a taxonomic revision of the hump-nosed pit viper (*Hypnale* sp.) considered to comprise three species: *H. hypnale*, common to Sri Lanka and the Western Ghats of peninsular India; and *H. nepa* and *H. walli*, both of which are endemic to Sri Lanka. *H. walli* is a junior synonym of *H. nepa*, and is restricted to the higher elevations of Sri Lanka's central mountains, while *H. zara* is distributed in the island's south-western 'wet-zone' lowlands. Envenoming by the latter two species is relatively infrequent, while *H. hypnale*, the widest distributed species in both Sri Lanka and the Western Ghats is responsible for most of the envenoming cases reported clinically (Ariaratnam *et al.*, 2008, 2009; Alirol *et al.*, 2010).

H. hypnale has a stout body build typical of viperids, but it is relatively slender compared to most other crotalids, and is small in size (body length rarely exceeds 0.5 m). It has a wide, triangle-shaped head with large frontal and parietal shields, while those on the snout are small and irregular. The snout is pointed and turned upwards, ending in a hump (Gloyd & Connant, 1990; Maduwage *et al.*, 2009). The color pattern is grayish with heavy brown mottling, overlaid with a double row of large dark spots. The ventral surface is brownish or yellowish with dark mottling, while the tip of the tail is yellow or reddish (Figure 2.6). The snake is mainly nocturnal and terrestrial. Its

existence is broad: it inhabits both wet and dry deciduous zones; can be found from secondary forests, hilly areas, various plantations to low land gardens and even latrines within home compounds. Though a slow mover, it easily turns aggressive if agitated, and is capable of making fast strikes. When confronted, it typically coils its body into C- or Z-shape, head raised along with hisses, and is capable of thrusting upward (where body left the ground momentarily) to attack. Being well camouflaged and tiny in size, it is also easily missed and trodden upon even in the day time, resulting in its defensive bite (Ariaratnam *et al.*, 2008).

H. hypnale, used to be in the *Agkistrodon* complex from the old systematics, was elevated to its current genus taxon based on morphological details and mitochondrial DNA analysis, which also confirmed its close phylogenetic relatedness to the Malayan pit viper, *Calloselasma rhodostoma* (Parkinson *et al.*, 1997; Vidal and Lecomte, 1998) as two basal Asiatic crotalids. The close phylogenetic relationship between *H. hypnale*, found in India subcontinent and *C. rhodostoma*, found in Southeast Asia, is a fascinating example of the existence of the ‘Malayan’ element in the fauna of Peninsular India. This phenomenon has been known since a long time, and the cause of these similarities have been explained by the Satpura Hypothesis (Hora, 1949), according to which fauna migration followed the Satpura trends of mountain through Peninsular India during the Pleistocene. The validity of Satpura Hypothesis, however, has been disputed later by Karanth (2003), who proposed the likelihood of a convergent evolution as the basis to the similarities observed. The phylogenetic information nevertheless suggests the possibility of similar venom properties of the two species, and the research potential in immunological profiling of the venoms.



Figure 2.6 An adult *Hypnale hypnale* The snake was a live male specimen kept at the serpentarium of the Faculty of Medicine, University of Colombo. approximately 0.25 m in length, with a stout body, wide head and a pointed snout that turns upwards, ending in a hump, and hence the common name hump-nosed pit viper. (Snake was photographed with permission from the serpentarium in-charge person, Prof. Christine A. G.)

Inset showed a picture taken from a dissection on a dead adult specimen of approx. 0.2 m in length. Note the relatively smaller size of the head, comparable to the proximal phalanx of the thumb. Exposed was the prominent venom gland (right) situated in the temporal region behind the eye.

2.8.2.2 Venomous bites and effects on human

Hypnale hypnale bites have long been noted for causing local tissue destructions, as reflected in its local name “kunakatuwa” which means “rotten bone” (another local name in Sinhalese is “polonthelissa” meaning viper with upturned lip). Local envenoming effects include hemorrhagic blistering, necrosis, pain and swelling as part of inflammation, and tender regional lymphadenopathy (commonly affected the inguinal lymph nodes). These local effects were well documented, however for more than a century it had been controversial as to whether *H. hypnale* bites could be fatal (Davy, 1821; Tennent, 1861; Wall, 1921; Deraniyagala, 1955; Sellahawa and Kumararatne, 1994). However, in a few of proven or suspected early cases of *H. hypnale* bites, systemic effects e.g. coagulopathy, microangiopathic hemolysis and renal failure have been observed (Varagunam and Panabokke, 1970; de Silva *et al.*, 1994; Premawardena *et al.*, 1996, 1998; Kularatne and Ratnatunga, 1999; Karunatilaka *et al.*, 2001). Recent clinical studies by Joseph *et al.* (2007) and Ariaratnam *et al.* (2008) authenticated the high prevalence and the severe complications of *H. hypnale* bites with an overall fatality rate of 1.7%.

Ariaratnam *et al.* (2008) reported that one-half of *H. hypnale* bites in a series of 302 cases occurred between 1800 h and midnight, while people were returning home from work and recreational activities. Approximately 50% of cases occurred within patient's compound (while walking, building, gardening, picking firewood, visiting wells or latrines), others occurred on roads or footpaths and at agricultural sites. Male patients outnumbered female in frequency of bites, and most bites (81%) were inflicted at the lower limbs (feet or ankles). Local effects with tissue destruction were common among the patients, with some severe cases needing amputation and skin grafting. Systemic envenoming was seen in approximately 40% of cases, characterized by hemostatic

dysfunction (coagulopathy, fibrinolysis, thrombocytopenia, spontaneous systemic hemorrhage), and acute kidney injury developed in about 30% of those showing hemostatic disturbances. Sunanda *et al.* (2010) reported a case where massive retroperitoneal hemotoma was only detected five weeks after *H. hypnale* bite, with lower limb deep vein thrombosis developed concurrently possibly secondary to the pressure effect of the hematoma. It appears that envenoming by *H. hypnale* typically results in local tissue destructions as described in most viperid bites, while the systemic complications are associated with varied degree of hemostatic disturbances that could culminate in a lethal hemorrhagic disorder and possibly complicate acute kidney injury in the victims (Sitprija, 2008).

2.8.2.3 Diagnosis, management and problems associated

In the Western Ghats of India and Sri Lanka, diagnosis of biting snake is often made either by identification of the snake brought to the hospital or based on description given by the victims. These are not always possible especially if the bite occurs in the dark or in bushes. In fact, frequent misidentification of *H. hypnale* as *Echis carinatus* or occasionally *D. russelii* had been reported (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008), even when the biting snakes were brought to the hospitals. This resulted in the administration of unwarranted, inappropriate antivenom, which was totally ineffective and costly, besides risking fatal complications, e.g. anaphylaxis. As such, Ariaratnam *et al.* (2009) proposed a syndromic approach to ascertain the cause of snake envenomation based on a step-wise clinical assessment on a series of envenoming features. The syndromic approach however may have certain limitations of use when a patient's clinical progress does not follow strictly the typical syndrome outlined. For instance, the development of acute kidney injury is a key feature to discern between *H. hypnale* and *E. carinatus* envenomings for a patient with hemostatic derangement (while

neurologically intact – ruling out *D. russelii* envenoming). However, acute kidney injury does not complicate all *H. hypnale* systemic envenomations; hence the absence of this anticipated complication may not totally rule in *E. carinatus* bite and does not warrant its antivenom. Nevertheless, coupled with patient's account, the syndromic approach mentioned currently represents the most useful clinical guide map for diagnosis in the absence of a reliable laboratory test for validating the biting species.

To date, despite the recent authentication of *H. hypnale* in causing severe envenoming, there is still no antivenom clinically available as effective treatment. Antivenoms available in the region are manufactured by Indian companies, raised against the historical Big Four, which have been proven ineffective in *H. hypnale* envenoming (Sellahewa *et al.*, 1995; Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008). *H. hypnale*-envenomed patients are therefore managed with supportive and ancillary treatments such as blood product transfusion, renal replacement therapy (usually peritoneal dialysis) and surgical intervention for necrotizing wounds, without the definite antidote, i.e. antivenom. In view of some of the envenoming effects that may develop slowly, e.g. prolonged coagulopathy and extensive tissue necrosis, antivenom should be given as early as possible to neutralize the venom in the body before its pathophysiological effects set far. Unfortunately, despite the lack of effective antivenom recognized (Ariaratnam *et al.*, 2008), research and production of an antidote against *H. hypnale* envenoming seemingly have not received much attention from most manufacturers, possibly due to market consideration and investment hindrance. The search for effective antivenom thus becomes an urgent effort to overcome the endemic medical crisis related to envenomation by *H. hypnale*.

Therefore, research works on the toxinological profiles of the venom of *H. hypnale* should address the following aspects:

- (1) The toxic effects of the venom, especially on the hematoxicity. Several toxins may be responsible for this; particularly noteworthy is the procoagulant or thrombin-like enzymes commonly present substantially in the venoms of vipers and pit vipers.
- (2) The composition of venom toxins and their correlations with the envenoming syndrome. Ascertaining the principal toxins is essential for clinical management and antivenom design.
- (3) Antivenom assessment for paraspecific protection in view of the lack of effective antivenoms in the region.
- (4) The immunological property of the venom and specific antibody production. This will have implications in the development of a diagnostic immunoassay as well as antivenom production in the future.

CHAPTER 3

General Materials and Methods

3.1 MATERIALS

3.1.1 Animals

Male Sprague Dawley rats (~150-300 g), albino mice of ICR-strain (~15-25 g) were supplied by the Laboratory Animal Center, University of Malaya. New Zealand White rabbits (~2 kg) were purchased from Chenur Supplies, Seri Kemabangan, Malaysia. The use of animals was approved by the institute [ethics clearance number: PM/03/03/2010/FSY(R)] and the animals were handled according to the guidelines given by the Council for International Organization of Medical Sciences (CIOMS) on animal experimentation (Howard-Jones, 1995). As the studies involved the use of venom that could induce great pain and distress in animals, the number of animals had been minimized ($n = 3-4$). Nevertheless, the experimental conditions and animal preparation were optimized to reduce variations, and the results were analyzed with appropriate statistical tests for significance (see Section 3.3.3 and Methods section in respective chapters).

3.1.2 Anesthesia

Xylazine and ketamine were supplied by Troy Laboratories, Australia. Diethyl ether was supplied by R & M Marketing, Essex, UK.

3.1.3 Snake venoms

Hypnale hypnale venom was a pooled sample obtained from the milking of >10 adult snakes captured in Sri Lanka (Gamapha, Kelaniya, Avissawela, Colombo regions). The snakes were kept at the serpentarium at the University of Colombo, Sri Lanka and were identified by Anslem de Silva, an expert herpetologist. *Daboia russelii* and *Echis carinatus sinhaleyus* venoms were obtained from the same source and the snakes were

captured in Sri Lanka (Anuradhapura, Ratnapura, Galle, Colombo regions for *D. russelii*; and Mannar for *E. carinatus sinhaleys*).

The following venoms were obtained from the respective suppliers: *Protobothrops flavoviridis*, *Protobothrops tokarensis*, *Cryptelytrops purpureomaculatus*, *Cryptelytrops albolabris*, *Tropidolaemus wagleri*, *Daboia siamensis* (Thai and Myanmar origins), *Naja nigricollis*, *Bungarus flaviceps*, *Bungarus candidus*, *Ophiophagus hannah*, *Naja naja* (Sri Lanka), *Naja sputatrix* (Java) from Latoxan (France); *Agkistrodon piscivorus leukostoma*, *Crotalus atrox* from Sigma (USA); *Crotalus viridis* from Miami Serpentarium (USA); *Popeia popeiorum*, *Viridovipera stejnegeri*, *Bothrops asper* from Ventoxin (USA); *Dendroaspis angusticeps*, *Pseudonaja textilis*, *Oxyuranus microlepidotus* from Venom Supplies (South Australia); *Calliophis bivirgatus* from a local snake handler (Malaysia), *Enhydrina schistosa* from Penang Snake Venom Institute (Malaysia).

3.1.4 Antivenoms

Monovalent Malayan pit viper antivenom (MPVA) (Batch no. CR00909, exp.date 2/11/2014) and Hemato polyvalent antivenom (HPA) (Batch no. HP00108, exp.date 6/11/2013) were free gifts from Queen Saobhva Memorial Institute, Thai Red Cross Society (TRCS). The Bharat Polyvalent ASVS (anti-snake venom serum) (Batch no.A5309049, exp.date 03/2013) was supplied by Bharat Serums and Vaccines, Mumbai, India.

3.1.5 Chemicals and consumables

Chromatography columns and media: Resource[®] Q ion-exchange column (6.4 mm x 30 mm, 1 ml gel volume, 15 µm particle size), HiTrap[™] Protein A-Sepharose gel affinity

column (1.6 x 2.5 cm, 5 ml gel column, 34 μ m particle size), Sephadex[®] G-25 gel filtration medium and Superdex[™] 200 HR 10/30 gel filtration column (10 x 300 mm, 13 μ m particle size) were from GE Healthcare, Uppsala, Sweden. LiChrospher[®] WP 300 C18 column (250 x 4 mm, approx. 5 ml gel volume, 5 μ m particle size) was from Merck KGaA, Darmstadt, Germany.

Nylon syringe filter with polypropylene housing (13 mm, 0.2 μ m) and filter papers grade 1 (retention size: 11 μ m; diameter: 180 mm) (both from Whatman Inc., USA), nitrocellulose filter (0.22 μ m, 47 mm) (Millipore, USA), mini iBlot[®] Transfer Stacks with PVDF membranes (Invitrogen[™], USA), 96-well flat bottom microtiter plates (Nunc, Denmark), Vivaspin[™] centrifugal concentrators (Sartorius Stedim Biotech, Germany), urine test strips (Combur[™] 10, Roche Diagnostics, Germany), disposable syringes (Terumo, Philippines), needles and U-100 insulin needles (Terumo, Japan) were purchased from the respective manufacturers.

Goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate and goat anti-equine IgG-HRP conjugate were supplied by Bio-Rad Laboratories, USA. Protein markers. i.e. Spectra[™] Multicolor Broad Range Protein Ladder (Thermo Scientific, USA), BIO-RAD SDS-PAGE Molecular Weight Standards Broad Range and BIO-RAD Gel filtration Standards (Bio-Rad Laboratories, USA) were supplied by the respective manufacturers. Acetonitrile (HPLC grade), ethanol, methanol and formaldehyde were from Friendemann Schindt Chemicals, Germany. Glycerol, hydrogen peroxide, acetic acid, phosphoric acid and sulfuric acid were from J. T. Baker, USA. Glycine (electrophoresis grade) and Tris were from MP Biomedicals, France. Thrombin of bovine origin prepared as 1000 unit/mL was from Parke-Davis, USA. All other chemicals and reagents utilized were of analytical grade supplied by Sigma, USA.

3.1.6 Common buffers

Filtered and deionized 'ultrapure' (Type 1) water was obtained from the ELGA PURELAB Option-Q water purification system (Veolia Water Solutions and Technologies, UK).

Phosphate-buffered saline (PBS; 1X)

PBS was prepared by dissolving 8.00 g of NaCl, 0.20 g of KCl, 2.68 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 0.24 g of KH_2PO_4 in 1 L ultrapure water and the solution was adjusted to pH 7.4.

Normal saline

Normal saline was prepared by dissolving 8.00 g of NaCl in 1 L ultrapure water and the solution was adjusted to pH 7.4.

3.2 GENERAL METHODS

3.2.1 Determination of protein concentration

Protein concentration was determined by the protein dye binding method according to Bradford (1976). In brief, the Bradford reagent (5X stock) containing 0.05% (w/v) Coomassie Brilliant Blue G-250, 25% (v/v) ethanol and 42.5% (v/v) phosphoric acid was prepared with ultrapure water and stored in an amber bottle stored at room temperature. One millilitre of the reagent (diluted 1:4 in ultrapure water) was added with 20 μL standards/protein samples and incubated at room temperature for 5 minutes prior to absorbance reading at 595 nm. A calibration curve was constructed from a series of diluted bovine serum albumin, prepared from a standard 1 mg/mL bovine serum albumin stock.

3.2.2 Electrophoresis

3.2.2.1 Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted in an electrophoresis (slab) system (Enduro PAGE System; Labnet International, USA) according to the principle of Laemmli (1970) and method as modified by Studier (1973). The following reagents or buffers were prepared:

Solution A: 29.2% (w/v) acrylamide, 0.8% (w/v) N-N'-methylene bisacrylamide, in ultrapure water.

Solution B: 1.5 M Tris-HCl buffer, pH 8.8, containing 0.4% sodium dodecyl sulfate (w/v).

Solution C: 10% (w/v) ammonium persulfate, freshly prepared.

Solution D: 0.5 M Tris-HCl containing 0.4% sodium dodecyl sulfate (w/v), pH 6.8.

Electrophoresis buffer: 0.025 M Tris, 0.192 M glycine, 0.1% sodium dodecyl sulfate, pH 8.3.

Reducing sample incubation buffer*: 62 mM Tris-HCl, pH 6.8, 2.3% sodium dodecyl sulfate (w/v), 10% (v/v) glycerol, 5% (v/v) beta-mercaptoethanol and 0.005% bromophenol blue.

Fixing solution: 40% (v/v) methanol, 10% (v/v) acetic acid in ultrapure water.

Staining solution: 0.2% (w/v) Coomassie Blue R-250 in the fixing solution, filtered.

Destaining solution: 5% (v/v) methanol, 7% (v/v) acetic acid in ultrapure water.

*For non-reducing sample incubation buffer, beta-mercaptoethanol was omitted.

3.2.2.2 Preparation of separating and stacking gels

The gel contents were prepared according to Table 3.1 (amount sufficient for 2 gels).

Table 3.1 Preparation of separating and stacking gels for SDS-PAGE.

Composition	12.5% separating gel	15% separating gel	4% stacking gel
Solution A (mL)	3.75	4.5	1.4
Solution B (mL)	3	3	-
Solution C (μL)	50	50	200
Solution D (mL)	-	-	2.5
Ultrapure water (mL)	2.25	1.5	6.1
TEMED* (μL)	25	20	10

*TEMED: tetramethylethylenediamine

For separating gel preparation, upon adding TEMED, the solution mixture was swiftly poured between the glass plates, up to approx. 2 cm below the notch. The slab gel size was 10 cm x 10 cm x 0.1 cm. Ultrapure water was layered on the gel to achieve an even surface. Polymerization occurred within 15 min and the water was poured off. For stacking gel preparation, upon adding TEMED, the solution mixture was layered on top of the polymerized separating gel. A comb was quickly inserted to create sample loading wells, and was left *in situ* for 20 min to ensure complete polymerization. Both the separating and stacking gels were prepared carefully to avoid formation of air bubbles within the gels.

3.2.2.3 Preparation and loading of protein samples

All protein samples were mixed with the sample incubation buffer in 1:1 volume ratio. Heating of the mixture in a boiling water bath for 15 min was needed for reducing SDS-PAGE. The mixture of protein sample with sample incubation buffer typically made up ~20-25 μL. Heated samples (for reducing SDS-PAGE) were left to cool at room temperature. All sample mixtures were briefly centrifuged to spin down any residues on the wall of the preparation tubes. The sample mixtures (and appropriate protein marker) were then separately pipetted and loaded onto the wells created in the stacking gel.

3.2.2.4 Running condition

The glass plates with gels formed within were assembled onto the electrophoresis chamber. The chamber were filled with the electrophoresis buffer and connected to a power supply. Electrophoresis was carried out with at a constant voltage of 80 volts for sample stacking (~14 min), followed by a constant voltage of 100 volts for sample separation (~2 h). The advancement of the front dye of sample was monitored. The electrophoresis was completed when the front dye of sample was approx. 1 cm from the bottom edge of the gel.

3.2.2.5 Fixing, staining and destaining

Upon completion of the electrophoresis, the plates were carefully opened to expose the sandwiched gel, which was then immersed in the fixing solution for 5 min prior to Coomassie Blue R-250 staining (for 15 min). The gel was destained in the destaining solution until clear.

3.2.2.6 Protein molecular mass calibration by SDS-PAGE

The \log_{10} molecular mass of the standard proteins was plotted against their respective R_f (relative mobility) to obtain a calibration curve. R_f was defined as the ratio of distance migrated by protein to the distance migrated by front gel. The following two sets of protein markers, available commercially, were used:

- (1) Spectra™ Multicolor Broad Range Protein Ladder (Thermo Scientific, USA): 10 proteins with molecular masses (kDa) of 10, 15, 25, 35, 40, 50, 70, 100, 140, 260;

(2) SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad, USA): 8 proteins with molecular masses (Da) of 7 100, 20 461, 29 054, 37 659, 51 783, 96 190, 115 281, 200 768.

3.3.3 Statistical analysis

Median lethal dose (LD₅₀) and median effective dose (ED₅₀) were expressed along with 95% confidence intervals (C.I.) according to probit analysis method of Finney (1952) using BioStat 2009 software (AnalystSoft Inc.). Statistical significance ($p < 0.05$) between mean values were analysed by unpaired Student's t-test or one-way ANOVA (with Tukey's post hoc test), using SPSS software.

CHAPTER 4

Enzymatic and Toxinological Activities of *Hypnale hypnale* Venom

4.1 INTRODUCTION

Snake venoms are mixtures of biologically active compounds (mainly proteins and peptides), with important roles in predation and digestion. The complex nature is unveiled by the fact that each component may target at one or more tissue receptors or biochemical pathways, causing profound alteration in the normal physiology of the envenomed. Defensive venomous bites on human result in envenoming syndromes that constitute a substantial health problem in many parts of the world. The problem becomes even more clinically challenging when knowledge of the venom's properties is limited and when specific treatment i.e. effective antivenom is not available. *H. hypnale* envenoming, which is prevalent and critical in Sri Lanka, shares the characteristics of such a clinical issue. In this chapter, the venom was studied from the point of milking, followed by a series of enzymatic as well as toxic profiling. The most important toxinological parameter, median lethal dose (LD_{50}), of the venom was established in laboratory mice for different routes of administration. The venom's SDS-PAGE profiling, its fractionation by anion exchange high performance liquid chromatography and biological testing of each fraction were also performed. The knowledge gained from studying fundamental biological properties of this venom is essential for a deeper understanding of its clinical syndrome, for venom neutralization study, and for the indication for further research of relevant toxins.

4.2 METHODS

4.2.1 Collection of the venom of *Hypnale hypnale* and the quantitation of average venom mass

Each individual snake (prior to weekly oral feeding) was removed carefully from its terrarium with a snake hook and placed on a flat surface. The snake head was pinned down with the hook that was quickly replaced by the index finger, while both sides of the head were grasped by the thumb and the middle finger just behind its eyes. Gentle massage was applied to the venom glands at the post-orbital regions while the snake was induced to bite once through a thin film stretched to cover a polystyrene collecting vial. Venom expelled through the fangs was collected in the vial for each snake and freeze-dried individually, following which its dry mass was determined (n = 6).

4.2.2 Separation of *Hypnale hypnale* venom by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Venoms of *H. hypnale*, *Calloselasma rhodostoma*, *Cryptelytrops purpureomaculatus*, *Tropidolaemus wagleri*, *Daboia russelii*, *Echis carinatus sinhaleus*, *Calliophis bivirgatus*, *Bungarus multicinctus*, *Naja sputatrix* (10 µg each) dissolved in distilled water and incubated with reducing incubation buffer at 1:1 ratio (total volume = 20 µL). The mixtures were then boiled at 100 °C for 10 minutes, cooled down and centrifuged briefly to gather the condensed liquid. The samples were loaded onto a 15% SDS polyacrylamide gel, and subjected to running condition as following: constant voltage of 80 volts in stacking phase, followed by constant voltage of 100 volts in separating phase. Upon completion, the gel was stained with Coomassie Blue R-250.

4.2.3 Fractionation of *Hypnale hypnale* venom by Resource[®] Q ion exchange high performance liquid chromatography

Five milligrams of the venom dissolved in 200 μ L of starting buffer (20 mM Tris-HCl, pH 8.5) was filtered with a 0.2 μ m pore size syringe filter. It was then injected into Resource[®] Q ion exchange column (6.4 mm x 30 mm, 1 mL gel volume, 15 μ m particle size, 200-10,000 Å, from GE Healthcare, Switzerland) pre-equilibrated with starting buffer, and subsequently eluted by a linear, 0.0 to 0.5 M sodium chloride gradient (0-30% from 5 to 30 min, followed by 30-100% from 30 to 55 min), at the flow rate of 1 mL/min. The solvent delivery and gradient formation over 60 min was achieved using the Shimadzu LC-20AD high performance liquid chromatography (HPLC) system. Protein peaks were monitored by measuring the absorbance at 280 nm. The protein content of each fraction was determined by Bradford method.

4.2.4 Determination of enzymatic activities

(This part of work was done on the venoms of *H. hypnale* and *Calloselasma rhodostoma* for comparison, as well as on the HPLC-isolated protein fractions of *H. hypnale* venom. Two independent experiments were carried out on each test for each sample (whole venom for the two snakes; and each HPLC fraction of *H. hypnale* venom.) The average results were reported.)

4.2.4.1 Protease assay

Protease activity was measured by modification of the method described by Kunitz (1947). One milliliter of 1% casein in 0.25 M sodium phosphate buffer, pH 7.75, and 50

μL of sample solution were incubated for 30 min at 37 °C. The reaction was terminated by adding 1 mL of 5% trichloroacetic acid. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was measured at 280 nm. One unit of protease activity was defined as an increase of one absorbance unit per hour at 280 nm.

4.2.4.2 Phospholipase A₂ assay

Phospholipase A₂ activity was determined by the acidimetric method (Tan and Tan, 1988). The egg yolk substrate suspension was prepared by mixing one part of chicken egg yolk, one part of 18 mM calcium (II) chloride, and one part of 8.1 mM sodium deoxycholate. The pH of the substrate suspension was adjusted to 8.0 with 1 M sodium hydroxide. The suspension was stirred to ensure good mixing. A hundred microliters of sample was added to 15 mL of the substrate suspension and the rate of decrease in pH was recorded using a pH meter (Sartorius, Germany). A decrease of 1 pH unit of the egg yolk suspension corresponded to 133 μmoles of fatty acids released.

4.2.4.3 Arginine ester hydrolase assay

Arginine ester hydrolase was assayed using α-benzoyl arginine ethyl ester as substrate (Collins and Jones, 1972). The assay mixture contained 0.95 mL of 0.8 mM substrate in 0.05 M Tris-HCl buffer, pH 7.8, and 50 μl of sample solution. The reaction was followed by measuring the rate of increase in absorbance at 255 nm. The difference in the extinction coefficient at 255 nm is 815 cm⁻¹·M⁻¹.

4.2.4.4 Phosphodiesterase assay

Phosphodiesterase activity was determined by a method modified from Lo et al. (1969). A hundred microliters of sample was added to an assay mixture containing 0.5 mL of 2.5 mM calcium bis-p-nitrophenylphosphate, 0.3 mL of 0.01 M magnesium sulfate and

0.5 mL of 0.17 M veronal buffer, pH 9.0. The hydrolysis of the substrate was followed by measuring the rate of increase of absorbance at 400 nm. The extinction coefficient is $8100 \text{ cm}^{-1} \cdot \text{M}^{-1}$.

4.2.4.5 Alkaline phosphomonoesterase assay

Alkaline phosphomonoesterase activity was determined by a method modified from Lo et al. (1969). A hundred microliters of sample was added to an assay mixture containing 0.5 mL of 0.5 M glycine buffer (pH 8.5), 0.5 mL of 10 mM p-nitrophenylphosphate and 0.3 mL of 0.01 M magnesium sulfate. The mixture was incubated at 37 °C for 30 min. At the end of the incubation period, 2 mL of 0.2 M sodium hydroxide was added and allowed to stand for 20 min at room temperature. The absorbance at 400 nm was then measured. The extinction coefficient is $18500 \text{ cm}^{-1} \cdot \text{M}^{-1}$.

4.2.4.6 5'-Nucleotidase assay

5'-Nucleotidase activity was determined using 5'-AMP as substrate (Heppel and Hilmore, 1955). A hundred microliters of sample was added to an assay mixture containing 0.5 mL of 0.02 M 5'-AMP (pre-adjusted to pH 8.5), 0.5 mL of 0.2 M glycine buffer, pH 8.5, and 0.1 mL of 0.1 M magnesium sulfate. The mixture was incubated for 10 min at 37 °C, and the reaction was terminated by the addition of 1.5 mL of 10% trichloroacetic acid. The ascorbic acid method (Chen et al., 1956) was used to determine the inorganic phosphate content. To the above mixture, 1 mL of ascorbic acid reagent, containing equal parts of 3 M sulfuric acid, 2.5% ammonium molybdate, 10% ascorbic acid and water was added. The mixture was left at room temperature for 30 min, and the absorbance at 820 nm was then measured. A standard curve was constructed using known concentrations of inorganic phosphate.

4.2.4.7 Hyaluronidase assay

Hyaluronidase activity was determined turbidimetrically (Xu et al., 1982). The assay mixture contained 0.45 mL of 0.2 M acetate buffer, pH 5.0, containing 0.15 M sodium chloride and 0.2 mg of human umbilical cord hyaluronic acid and 50 µl of sample solution. The mixture was incubated for 1 h at 37 °C. Reaction was terminated by the addition of 1 mL of 2.5% cetyltrimethyl-ammonium bromide in 2% sodium hydroxide solution. After 30 min the absorbance at 400 nm was measured, and the enzymatic activity was expressed as NFU/mg (NFU = National Formulary Units). Appropriate dilutions of sample were made to ensure that the difference in absorbance between the blank and sample was less than 0.4. Hyaluronidase (EC 3.2.1.35) of bovine testes origin, purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) was used as standard, its activity is 270 National Formulary Unit (NFU) per mg. Various dilutions of hyaluronidase representing a series of NFUs were tested on solution containing 0.2 mg hyaluronic acid. The absorbance values were plotted against the NFUs as calibration.

4.2.4.8 L-amino acid oxidase

L-amino acid oxidase activity was determined as described in Tan *et al.* (1986). Fifty microliters of horseradish peroxidase (100 purpurogalin unit/mg) was added to 0.9 mL of 0.2 M triethanolamine buffer, pH 7.6, containing 0.1% L-leucine and 0.0075% o-dianidisine and incubated for 3 min at room temperature. Sample solution (50 µl) was then added and the increase in absorbance at 436 nm was measured. The molar absorption coefficient is $8.31 \times 10^3 \text{ cm}^{-1} \cdot \text{M}^{-1}$.

4.2.5 Determination of lethality, hemorrhagic, procoagulant and necrotic Activities

4.2.5.1 Median lethal dose (LD₅₀)

The LD₅₀ values of *H. hypnale* venom were determined for three different routes of injection (intravenous, intraperitoneal, intramuscular) of the venom into mice (n = 4 per dose) whose survival ratio was recorded after 48 hours. The sites of *i.v.*, *i.p.* and *i.m.* injections were, respectively, caudal vein, left lower abdominal quadrant, left or right quadriceps muscles. The LD₅₀ (and the 95% confidence intervals, CI) of the venom was calculated using the Probit analysis method of Finney (1952).

4.2.5.2 Procoagulant activity

Procoagulant activity was determined by the method described by Bogarin *et al.* (2000). The venom (50 µg in 0.1 mL) or venom fraction (0.1 mL) was added to 0.2 mL of bovine fibrinogen solution (2 g/L) at 37°C. The coagulation time (time required for fibrin strands to form) was recorded. A shorter coagulation time suggests higher procoagulant activity.

4.2.5.3 Hemorrhagic activity

Hemorrhagic activity was determined according to Theakston and Reid (1983) with modification. The venom dissolved in phosphate-buffered saline (50 µg in 50 µL) or the venom fraction (50 µL) was injected intradermally into the shaved dorsal skin of lightly anesthetized (by diethyl diether) mice. After 90 minutes, the animals were euthanized with an overdose of diethyl ether. The dorsal skin was removed carefully, and the mean diameter of the hemorrhagic lesion on the inner surface of the skin was measured.

4.2.5.4 Necrotic activity

Necrotic activity was determined by a modified version of the method of Theakston and Reid (1983). The venom (50 µg in 50 µL) or venom fraction (50 µL) was injected intradermally into the shaved dorsal skin of lightly anesthetized (with diethyl diether) mice. After 72 hours, the animals were euthanized with an overdose of diethyl ether, the dorsal skin was removed, and the mean diameter of the necrotic lesion was measured.

4.2.6 Fibrinogen clotting activity

The fibrinogen clotting activity (procoagulant effect) of *H. hypnale* venom was tested further on fibrinogens from several mammalian sources. Venoms from two other Asiatic pit vipers, *C. rhodostoma* and *C. purpureomaculatus*, well known for their procoagulant effects (Tan and Ponnudurai, 1996; Tan, 2010) were studied simultaneously for comparison.

The venom (50 µg in 0.1 mL) was added to 0.2 mL of fibrinogen solution from various animal sources (2 g/l) at 37 °C. The coagulation time (time taken for fibrin strands to form) was recorded. To study the time course of venoms procoagulant activities, each venom (50 µg in 0.1 mL) was added to 0.2 mL of fibrinogen solution (2 g/l) preincubated at 37 °C. The absorbance of the mixture at 450 nm was read up to 10 minutes to monitor the formation and degradation of fibrin clots.

4.3 RESULTS

4.3.1 Collection and quantitation of *Hypnale hypnale* venom

Lyophilization left the venom milked from *H. hypnale* in yellowish crystalline powder form. In the investigation of venom yield, the milking of six adult *H. hypnale* snakes yielded 10.0 mg, 8.7 mg, 11.4 mg, 20.0 mg, 12.2 mg and 15.6 mg, individually, of lyophilized venom. The average amount of venom obtained per milking is therefore 13 mg (± 4.2 mg).

4.3.2 SDS-PAGE profiling of *Hypnale hypnale* and several Asiatic snake venoms

The SDS-PAGE showed that the venoms of *H. hypnale* and several Asiatic vipers and pit vipers (except *Tropidolaemus wagleri*) contain predominantly moderate-to-high molecular mass proteins. In contrast, proteins of low molecular masses are abundant in the elapid venoms with relatively lesser high molecular mass proteins (except *Ophiophagus hannah*) (Figure 4.1).

4.3.3 Lethality of *Hypnale hypnale* venom

The lethality of the venom was expressed in term of median lethal dose (LD₅₀), with comparison made among the different routes of administration. The intravenous, intraperitoneal and intramuscular median lethal doses of *H. hypnale* venom were, respectively, 0.90 µg/g (95% CI: 0.42-1.84 µg/g), 6.0 µg/g (95% CI: 4.20-8.10 µg/g) and 13.7 µg/g (95% CI: 8.44-19.50 µg/g) of mouse body weight.

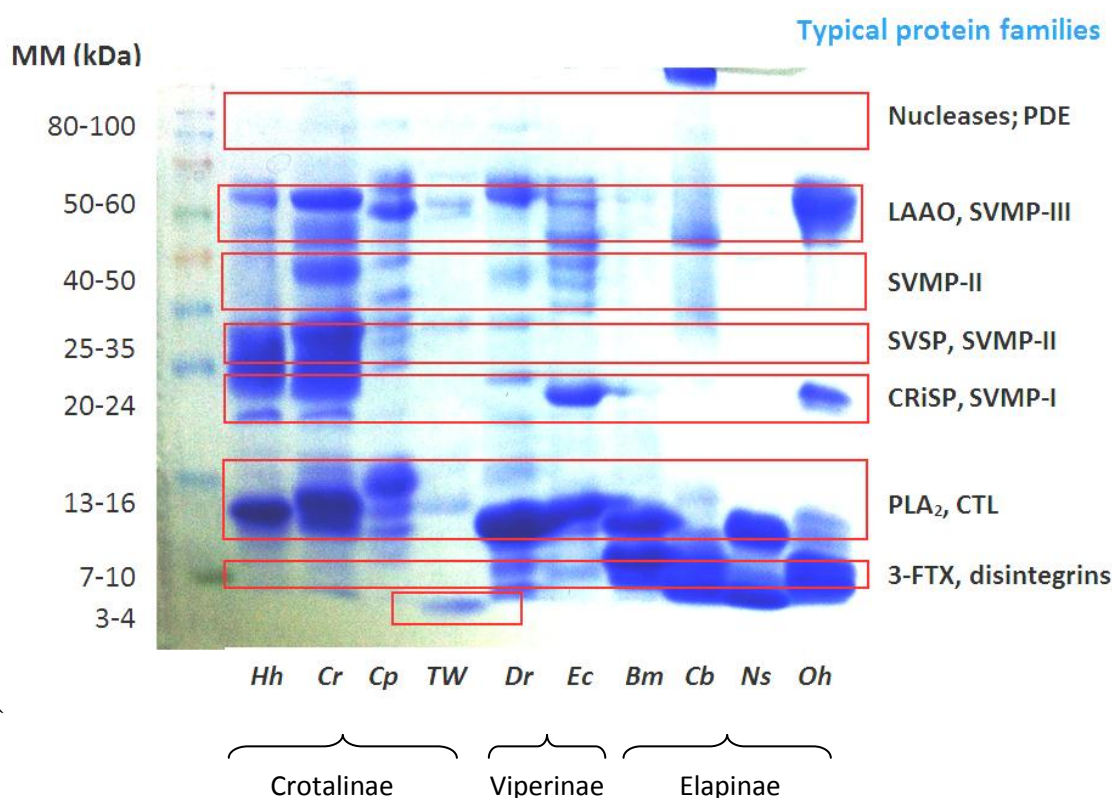


Figure 4.1 SDS-PAGE of the venoms of *Hypnale hypnale* and several Asiatic snakes. The venoms (10 µg each) were loaded on a 15% SDS polyacrylamide gel and electrophoresed under reducing condition. Left panel shows the typical possible protein families found in the venom; right panel shows the molecular weights; lower panel shows the species of the snakes with the subfamilies they belong to.

Hh: *Hypnale hypnale*; *Cr*: *Calloselasma rhodostoma*; *Cp*: *Cryptelytrops purpureomaculatus*; *Tw*: *Tropidolaemus wagleri*. (Crotalinae)

Dr: *Daboia russelii*; *Ec*: *Echis carinatus*. (Viperinae)

Bm: *Bungarus multicinctus*; *Cb*: *Calliophis bivirgatus*; *Ns*: *Naja sputatrix*;

Oh: *Ophiophagus hannah*. (Elapinae)

PDE: phosphodiesterase, LAAO: L-amino acid oxidase, SVMP: snake venom metalloproteases, SVSP: snake venom serine proteases, CRiSP: cysteine-rich secretory proteins, PLA₂: phospholipase A₂, CTL: C-type lectin, 3-FTX: three-finger toxin, MM: molecular mass.

4.3.4 Biological properties of *Hypnale hypnale* and *Calloselasma rhodostoma* Venoms

H. hypnale venom exhibited enzymatic properties similar to *C. rhodostoma* venom (Table 4.1), which are quite typical of pit viper venoms, notably the presence of arginine ester hydrolase and potent protease activities.

H. hypnale venom also showed potent procoagulant or thrombin-like enzyme activities (lower than that of *C. rhodostoma*), as well as hemorrhagic and necrotic properties (higher than or similar to that of *C. rhodostoma*).

4.3.5 Fractionation of *Hypnale hypnale* venom by Resource[®] Q anion exchange chromatography

With Resource[®] Q column, an anion exchange HPLC column, *H. hypnale* venom was resolved with various NaCl concentrations at pH 8.5 to yield twelve major fractions (Figure 4.2). Protein determination by the Bradford method showed that fraction 12 has very little protein content. Fractions 1 and 2 were unbound fractions and contained basic proteins, while the remaining fractions generally contained proteins of an increasingly acidic nature.

Table 4.1 Enzymatic, hemorrhagic and necrotic activities of *Hypnale hypnale* and *Calloselasma rhodostoma* venoms.

Activity Species	PRO	PLA ₂	LAO	AEH	PME	PDE	5'-Nuc	HYA	TLE	HEM	NEC
<i>H. hypnale</i> [#]	15.8	191.5	191.7	10.05	4.8	4.1	1.8	115.5	20	23	7.5
<i>C. rhodostoma</i> [#]	18.5	79.8	165.4	8.96	4.6	18.5	1.9	76.6	10	16	8.0
<i>H. hypnale</i> [@]	7.8	126	110	15.2	12	6	2.7	85	22	1.6*	ND

PRO: protease, unit/mg; PLA₂: phospholipase A₂, $\mu\text{mole}/\text{min}/\text{mg}$; LAO: L-amino acid oxidase, $\text{nmole}/\text{min}/\text{mg}$; AEH: arginine ester hydrolase, $\mu\text{mole}/\text{min}/\text{mg}$; PME: alkaline phosphomonoesterase, $\text{nmole}/\text{min}/\text{mg}$; PDE: phosphodiesterase, $\text{nmole}/\text{min}/\text{mg}$; 5'-Nuc: 5'-nucleotidase, $\mu\text{mole}/\text{min}/\text{mg}$; HYA: hyaluronidase, NFU/mg; TLE: thrombin-like enzyme (procoagulant enzyme), s/50 μg venom; Hem: hemorrhagin, mm/50 μg venom; NEC: necrosis, mm/50 μg venom; ND: not determined. Values were the average results from two independent experiments.

[#] Present study; [@] reference to Tan and Ponnudurai (1996); * the unit was expressed as the product of two perpendicular diameters of the lesion, which is different from the unit defined in the present study.

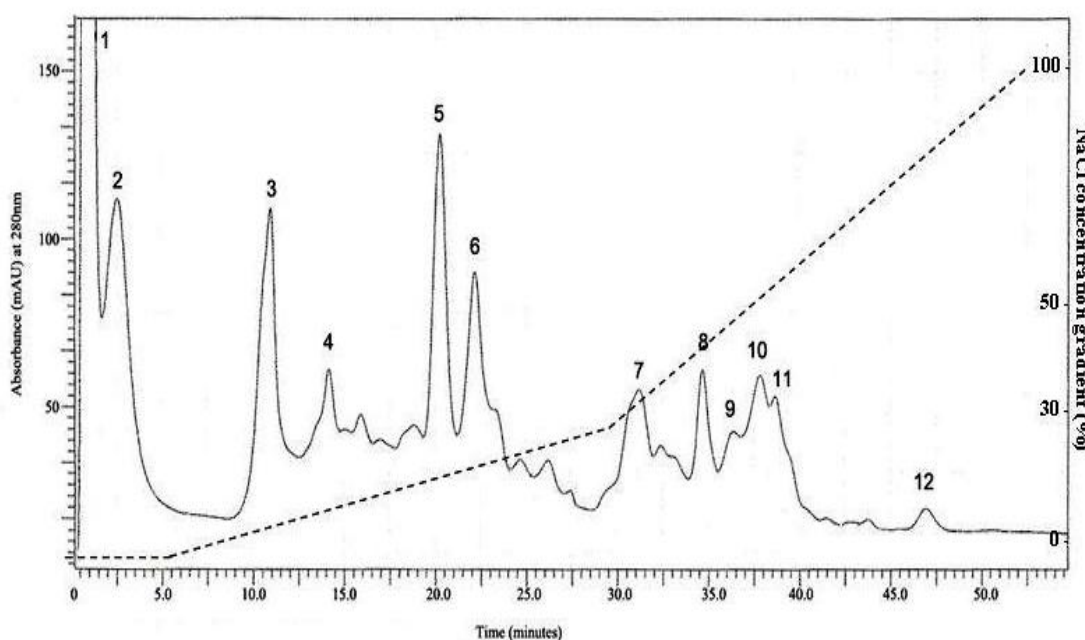


Figure 4.2 Fractionation of *Hypnale hypnale* venom with Resource® Q anion exchange chromatography. The venom (5 mg) was injected into the column equilibrated with 20 mM Tris-HCl, pH 8.5, and eluted by a linear, 0.0 to 0.5 M NaCl gradient (0-30% from 5 to 30 minutes, followed by 30-100% from 30 to 55 minutes. Flow rate was 1 mL/min. (-----): NaCl concentration gradient (%).

4.3.6 Biochemical properties of the protein fractions of *Hypnale hypnale* venom

Table 4.2 shows the biological properties of the 12 fractions obtained from Resource® Q chromatography of *H. hypnale* venom. Fraction 1, containing the basic proteins, was certainly heterogeneous, as it possessed multiple enzyme activities including proteases, phospholipase A₂, alkaline phosphomonoesterase, phosphodiesterase and 5'-nucleotidase activities. It was also the fraction that exhibited remarkable hemorrhagic and necrotic activities. Other enzymatic properties were detected in the following acidic fractions, with generally 1-3 enzymes co-eluted in a fraction. Hyaluronidase and L-amino acid oxidase appeared to be highly acidic proteins present in the venoms.

Table 4.2 Enzymatic, hemorrhagic and necrotic activities of major protein fractions of *Hypnale hypnale* venom obtained from Resource® Q ion exchange chromatography.

Activity Fraction	AMT	PRO	PLA ₂	LAO	AEH	PME	PDE	5'-Nuc	HYA	TLE	HEM	NEC
1	240	8.3	79.8	–	–	0.5	9.8	0.18	–	–	9	6
2	59	–	–	–	–	–	–	–	–	470	–	–
3	77	–	159.6	–	–	–	–	–	–	212	–	–
4	32	–	–	–	–	–	–	0.15	–	115	–	–
5	47	–	–	–	9.8	–	–	0.30	–	84	–	–
6	58	–	–	–	2.5	–	–	0.66	–	77	–	–
7	28	–	–	–	–	–	–	–	–	146	–	–
8	18	–	–	–	–	1.7	–	–	–	610	–	–
9	12	–	–	13.8	–	0.9	–	–	6.9	–	–	–
10	41	–	–	11.3	–	0.1	–	–	46.9	–	–	–
11	10	–	–	–	–	–	–	–	6.6	–	–	–
12	4	–	–	–	–	–	–	–	–	–	–	–

AMT: Total protein amount recovered in each respective fraction, µg; PRO: protease, unit/mL; PLA₂: phospholipase A₂, µmole/min/mL; LAO: L-amino acid oxidase, nmole/min/mL; AEH: arginine ester hydrolase, µmole/min/mL; PME: alkaline phosphomonoesterase, nmole/min/mL; PDE: phosphodiesterase, nmole/min/mL; 5'-Nuc: 5'-nucleotidase, µmole/min/mL; HYA: hyaluronidase, NFU/mL; TLE: thrombin-like enzyme (procoagulant enzyme), s/100 µL; HEM: hemorrhagic activity, mm/50 µL; NEC: necrotic activity, mm/50 µL, –: absent activity. Values were the average results from two independent experiments.

4.3.7 Fibrinogen clotting activity

The clotting activity of *H. hypnale* venom showed distinct species differences. Table 4.3 shows the procoagulant effect of the three different pit viper venoms measured in clotting time, while Figure 4.3 illustrated the time course of the procoagulant activity. Venoms of *C. rhodostoma* and *C. purpureomaculatus* were included in the assays for comparison purpose.

Table 4.3 Procoagulant effects of venoms from three pit vipers on mammalian fibrinogens.

Venom	<i>H. hypnale</i>	<i>C. rhodostoma</i>	<i>C. purpureomaculatus</i>
Source of fibrinogen	Clotting time* (s) (mean \pm SEM)	Clotting time (s) (mean \pm SEM)	Clotting time (s) (mean \pm SEM)
Rabbit	18.3 \pm 0.9	30.7 \pm 1.2	Turbidity > 120 s
Horse	42.2 \pm 1.0	40.1 \pm 0.6	Turbidity > 60 s
Dog	Turbidity** > 180 s	Turbidity > 180 s	Turbidity > 80 s
Goat	30.3 \pm 0.9	33.0 \pm 0.6	71.0 \pm 0.6
Bovine	14.3 \pm 0.3	11.3 \pm 0.9	56.7 \pm 0.7
Human	32.7 \pm 0.7	28.4 \pm 0.8	75.0 \pm 0.6

* Clotting time is defined as the time (s) for apparent fibrin strands to appear in the mixture of venom and fibrinogen.

** Turbidity refers to cloudiness appearing in the mixture of venom and fibrinogen, which may be interpreted as the formation of fibrin clots.

Results are expressed as the mean \pm SEM from three experiments.

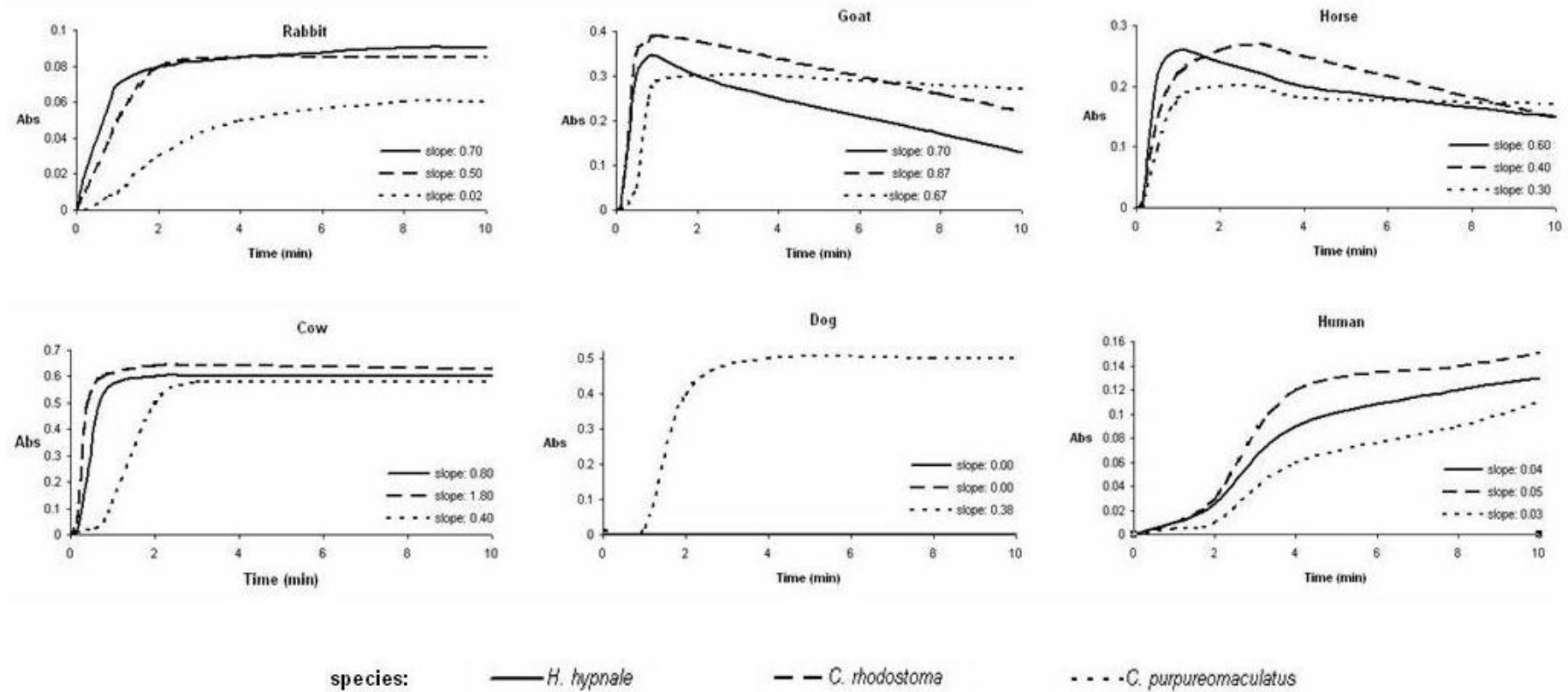


Figure 4.3 Time course of thrombin-like enzyme (procoagulant) activity of three different crotalid venoms on fibrinogens from several mammals (indicated on graph). The slope (gradient) for each curve is calculated from the steepest upward portion of the graph representing the maximal clotting rate expressed as absorbance/minute. Absorbance at 450 nm reflects the change in mixture clarity, which is responsible for the formation of fibrin strands as well as their dissolution/degradation. “Clot” used in this discussion represents the formation of fibrins.

4.4 DISCUSSION

4.4.1 Collection and estimation of *Hypnale hypnale* venom average amount

The maximum amount of venom injected per bite by *H. hypnale* is estimated to be around 10-20 mg, much lower than the approximately 50 mg per venom milking reported for its sister taxon, *C. rhodostoma* (Tan and Ponnudurai, 1996). This is to be expected as the body size of *H. hypnale* is small and rarely grows beyond 0.5 m (Figure 2.3). This information has a practical implication in pharmacokinetic study of the venom, and in neutralization study where the usefulness of an antivenom (if available) is evaluated according to the venom amount injected.

The prominent yellowish color of the venom, as observed in the venoms of *C. rhodostoma* and *Ophiophagus hannah*, suggests high content of L-amino acid oxidases (LAAOs) due to the presence of flavin coenzymes in LAAOs (Tan and Fung, 2010). Indeed, the venom exhibited high LAAO activity (Section 4.3.4), and a proteomic study revealed the composition of LAAOs as high as 20% of dried venom mass (Section 10.3.2.1).

4.4.2 Comparison of SDS-PAGE profiles of venoms

The venoms of vipers and pit vipers generally contain toxin proteins of higher molecular mass, which include metalloproteases (20-60 kDa depending on the class), cysteine-rich secretory proteins (20-25 kDa), serine proteases (25-40 kDa), L-amino acid oxidase (50-60 kDa), hyaluronidase (70-80 kDa), nucleases (80-90 kDa) and so on, in addition to medium molecular mass proteins such as phospholipase A₂ and C-type

lectins (13-15 kDa), as well as less abundant disintegrins of low molecular mass (6-7 kDa) (Mackessy, 2010a). The predominance of the high molecular mass toxins, typically the proteolytic and hydrolytic enzymes such as metalloproteases and serine proteases in these venoms, correlates well with the predominant pathology and clinical syndrome in envenomation by these Viperinae and Crotalinae snakes: hemorrhagic syndrome with deranged hemostasis and consumptive coagulopathy, extensive local tissue destruction and necrosis; while in some advanced cases, complications involving target organs such as pituitary infarct (*Daboia russelii*) and acute kidney injury occur (*Daboia russelii*, *H. hypnale*) (Than-Than et al., 1989; Warrell, 1989; de Silva et al., 1994; Ariaratnam et al., 2008). The venom of *Tropidolaemus wagleri*, however, is atypical among the other pit vipers as its principal toxins being vasoactive or neurotoxic polypeptides of very low molecular mass (~3 kDa), and there is very low abundance of high molecular mass hemotoxic proteases (Tan and Tan, 1989; Weinstein et al., 1991). On the other hand, the Elapinae venoms generally contain abundant low molecular mass toxins that belong to three-finger toxin family (~7 kDa), with potent neurotoxic and cytotoxic properties (Kini, 2002; Fry et al., 2003b). The venom of *Ophiophagus hannah* (king cobra), however, also contains large amount of high molecular mass proteins, majority of which are known to be L-amino acid oxidases (Tan and Fung, 2010).

4.4.3 Lethality of the venom

The variations in the median lethal doses (LD₅₀) among the three routes of administration (*i.m.*, *i.p.*, *i.v.*) suggest that the potency of toxins causing lethality differs depending on the routes of administration. A much lower intravenous LD₅₀ indicates that the venom is likely to be principally hemotoxic in view of its instant procoagulant action causing thromboembolic events, while the intraperitoneal LD₅₀ is higher than the

intravenous value possibly due to delayed and/or incomplete absorption (as in first-pass phenomenon) of venom toxins before reaching the systemic circulation (Ghosh, 1984; Turner, 1965). A relatively lower intraperitoneal LD₅₀ value compared to the intramuscular value suggests a higher bioavailability of the venom readily absorbed from the peritoneum with its larger surface area and richer blood supplies. Furthermore, intraperitoneal route would avoid the potential of venom components binding and interacting with muscles and dermal tissues, which might disrupt the toxin absorption as tissue and vasculature damages ensue. The *i.v.* LD₅₀ reported herein (0.9 µg/g) is lower than an earlier report of 65.4 µg per mouse by Ariaratnam et al., (2008), which was approximately equivalent to 3 µg/g (assuming mouse body weight to be 20 g). The difference could be due to either geographical or individual variation of the snake samples, which are known sources of variations in venom composition (Mackessy, 2010a).

It is also interesting to note that median lethal doses may be slightly varied from one laboratory to another. Based on unpublished observation, the different sizes of needle used for *i.v.* injection could technically cause variation in the determination of LD₅₀, as a 'larger bore' (relative to the diameter of mouse vein) hypodermic needle (< 27 gauge) potentially caused extravasation or wastage of content when injection was done via the caudal vein of mouse. Such technical variations should however be minimized using a more standardized protocol, to be established especially between laboratories working on a similar project.

4.4.4 Biological properties of the venoms

The *Hypnale hypnale* venom used in this study showed higher activities of proteases, phospholipase A₂, L-amino acid oxidase and hyaluronidase than those found previously by Tan and Ponnudurai (1992) who used commercially available venom source from an American serpentarium. This may be due to ontogenic differences as the venom used in this study was a recently pooled sample from snakes in their native country. The procoagulant activity, however, did not differ markedly between the two reports.

Venoms of *H. hypnale* and *C. rhodostoma* exhibited prominent and comparable procoagulant, hemorrhagic and necrotic activities, which are hallmarks of the pathophysiology induced by viperid venoms. The general similarities in the biological activities of *H. hypnale* venom and *C. rhodostoma* venom are also anticipated as they are closely related phylogenetically, and hence the possibility of sharing similar venom antigens (Parkinson et al., 1997; Vidal and Lecointre, 1998). Particularly noteworthy is the substantially higher phospholipase A₂ activity in *H. hypnale* venom in comparison to *C. rhodostoma* venom which usually exhibits rather low phospholipase A₂ activity. Phospholipases A₂ are known to exhibit multiple pharmacological activities, including lipid membrane damage, myotoxicity and myonecrosis (Kini, 1997, 2003). Substantial amounts of phospholipase A₂, proteases and L-amino acid oxidase, known for their cytotoxic and tissue-damaging properties, are likely responsible for the local envenoming features, e.g. edema, necrosis and hemorrhagic blistering, commonly seen after *H. hypnale* bites (Kini, 2003; Fox and Serrano, 2005; Joseph et al., 2007; Ariaratnam et al., 2008; Tan and Fung, 2010). In addition, hyaluronidase that presents substantially in the venom can synergistically potentiate the venom propagation and its local effects, contributing to severe local tissue destruction (Girish et al., 2004).

4.4.5 Biochemical properties of the HPLC protein fractions of *Hypnale hypnale* venom

Ion exchange chromatography separates proteins based on their ionic charges. It has been useful in snake venom fractionation as it does not affect the biological activity of eluted proteins, hence allowing their characterizations (Tan et al., 1986; Tan, 2010). The hemorrhagic and necrotic activities of fraction 1 were likely associated with its high proteolytic activity, as reported for viperid metalloproteinases (Fox and Serrano, 2005). Both fractions 1 and 3 exhibited phospholipase A₂ activity, suggesting that the venom contains at least two forms of phospholipase A₂. This finding is in accord with a previous report (Wang et al., 1999b), where only two out of four PLA₂ isoforms isolated from *H. hypnale* venom, using combined gel filtration and reverse-phased chromatography, exhibited enzymatic activities. We suggest that these two reported enzymes belong to a basic form (fraction 1) and an acidic form (fraction 3), respectively. The other two isoforms, suggested relating to myonecrotic pathology by Wang et al. (1999b), were not detected by the current enzymatic micellar assay but revealed in the proteomic study described in Chapter 10.

Hydrolytic enzymes – such as phosphodiesterase (a nuclease), 5' nucleotidase and alkaline phosphomonoesterase – are high molecular weight proteins known to be widely distributed across many snake taxa, but their toxic effects have been less extensively characterized partly due to the fact that these enzymes share similar substrates and biochemical properties (Dhananjaya et al., 2009). In general, the present results showed that all three enzymes were found in *H. hypnale* venom in appreciable amounts, particularly phosphodiesterase, which is usually abundant in crotalid venoms. Increase in phosphodiesterase activity had been associated with a drop in mean arterial pressure

and with locomotor depression, presumably due to reduced cAMP levels (Russell et al., 1963). This feature, however, has not been reported in systemic envenomation by *H. hypnale*, implying that its function might be more digestive than purely toxinological. The chromatography result also showed that 5'-nucleotidase and phosphomonoesterase from *H. hypnale* venom exist in both basic and acidic isoforms. It is intriguing to note the existence of acidic phosphomonoesterase in this venom, as this enzyme, contrary to the basic/alkaline phosphomonoesterase, and so far only been purified from sea snake venoms and has rarely been reported in other snake venoms (Uwatoko-Setoguchi, 1970).

Thrombin-like activity was found to be widespread from fraction 2 to fraction 8, indicating that the enzyme likely existed in multiple isoforms. The two fractions (fraction 5, 6) with the strongest thrombin-like activity were also the two fractions that hydrolyzed arginine esters, suggesting that the thrombin-like enzymes exhibited arginine ester hydrolase activity, a property similar to that of ICR-50 arvin, a coagulant enzyme derived from *C. rhodostoma* venom (Collins and Jones, 1972). The enzyme esterolytic activity towards *N*- α -benzoyl-L-arginine ethyl ester indicated its specific binding to the arginine residues in its substrate.

The venom also contained one or more forms of L-amino acid oxidase that appeared to be more acidic than many other enzymes, and its activity was noted to be lost on freezing. Such freeze-labile activity had been reported and the mechanism is presumably due to a limited conformational change of the enzyme structure (Curti et al., 1968; Iwanaga and Suzuki, 1979). Fraction 12, which contained few proteins, was found to be devoid of enzymatic activities. As demonstrated, venom fractionation helps elucidate the different components in venoms allowing their toxic characterizations for

pathophysiological correlations. Nonetheless, it also opens the door to manipulating specific toxins in search of potential therapeutic compounds, as already observed in drug discoveries of anacard, captopril etc. (Koh et al., 2006).

4.4.6 Fibrinogen clotting activity

The species-specificity of *H. hypnale* venom action on different mammalian fibrinogens was indicated by the differences in the clotting times (in increasing order as follows: bovine < rabbit < goat < human < horse < < dog). The clotting activity of *C. rhodostoma* venom exhibited a similar pattern (most and least active towards the bovine and dog, respectively) indicating that the thrombin-like activity of the venoms of these two phylogenetically related crotalids exhibited similar specificity to some extents towards fibrinogens. On the other hand, the specificity of the clotting activity of *C. purpureomaculatus* venom (bovine < goat < human < horse < < rabbit, dog) was somewhat different, and generally showed a lower clotting potency for bovine, goat and human fibrinogens.

The clotting activities of the three venoms were also monitored spectrophotometrically where the turbidity of the reaction mixture was continuously monitored for absorbance at 450 nm. The investigation of the time course of clot assembly had also been described by Pirkle *et al.* (1986). Generally, the appearance of fibrin or clots would result in turbidity, while degradation of the clots would reduce this property. Figure 4.3 shows that both *H. hypnale* and *C. rhodostoma* venoms caused rapid clotting with fibrinogens from bovine, goat, horse and rabbit specimens. Dissolution of clots was rapid in the case of horse and goat fibrinogens (turbidity reduced from 30 seconds). Both venoms were active in the clotting of human fibrinogen, showing a slow but

prolonged clotting effect. *C. purpureomaculatus* venom generally demonstrated moderate to low clotting activities, except for the case of dog fibrinogen where it appeared more active than the venoms of *H. hypnale* and *C. rhodostoma*. It is therefore suggested that a rapid clotting profile implies the possibility that the venom may precipitate acute thrombotic syndrome that can be instantly fatal, as observed in an experiment where a rabbit was injected with *H. hypnale* venom intravenously (unpublished data). In cases where clot formation is less rapid, or is accompanied by a concurrent degradation, the most likely cause of death would be a consumptive coagulopathy that gradually affects hemostasis, causing prolonged bleeding and a “slower” death. This is consistent with clinical features reported in cases of envenomation by *C. rhodostoma* (Reid et al., 1963; Ho et al., 1986a) and *H. hypnale* (Joseph et al., 2007; Ariaratnam et al. 2008). Deranged hemostasis leading to systemic bleeding has been a major feature in systemic envenomation by these crotalids. This severe, potentially fatal complication might only manifest itself following the bite as the preexisting coagulant factors get depleted or rendered inactive by the venom more rapidly than their production, hence tilting the hemostatic balance towards a tendency to bleed. A recent example was that of an extensive retroperitoneal hemorrhage only detected five weeks after a bite by *H. hypnale* in an adult patient (Sunanda et al., 2010). The findings indicated that frequent and extended monitoring of patient’s clotting profile may be necessary clinically; while at laboratory setting, the procoagulant enzyme from *H. hypnale* venom should be further isolated and characterized.

CHAPTER 5

In Search of Antivenom:

***In Vitro* and *In Vivo* Neutralization of *Hypnale hypnale* Venom**

5.1 INTRODUCTION

A pertinent issue surrounding the medical urgency of *Hypnale hypnale* envenomation in Sri Lanka and southwestern coast of India (Kerala) is the lack of a clinically effective antivenom. Locally available polyvalent antivenoms failed to cross-neutralize the venom (Sellaheewa *et al.*, 1995; Premawardena *et al.*, 1996, 1998; Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008), and to date there is still no update about specific antivenom production to be undertaken by any pharmaceutical company. Initiating antivenom production is known to require intensive research and long-term monetary investment on various aspects: animal (horse) farming and veterinarian care, expertise solicitation, laboratory facility for blood product processing and immunobiological testing, product quality control, antivenom packaging, storage, distribution, marketing and so on; hence, the expensive charges of antivenom products incurred worldwide. Unfortunately, the target markets - mostly developing and under-developed third world countries, are unlikely to promise a profitable return. The most practical solution therefore would be one that depends on existing heterologous effective antivenom so that the cost of production could be shared and minimized. In this study, we surveyed existing antivenoms outside the endemic area for cross-neutralization potential based on the likelihood of venom similarity of the snakes. Two recent products of the Thai Red Cross Society, monovalent Malayan pit viper antivenom and Hemato polyvalent antivenom, were chosen as the Malayan pit viper and *H. hypnale* are closely related phylogenically. They were tested for neutralizing potential on the lethal and various toxic effect of *H. hypnale* venom, both *in vitro* and *in vivo* in a mouse model.

5.2 METHODS

5.2.1 Determination of lethal and toxic doses of *Hypnale hypnale* venom

5.2.1.1 Determination of median lethal dose (LD₅₀) and minimal lethal dose (MLD)

The *i.v.* and *i.m.* LD₅₀ values of *H. hypnale* venom were determined as described in Chapter 4 (see 4.2.5.1). The *i.m.* MLD (minimum lethal dose) of the venom is defined as the minimal venom dose that killed all the mice tested ($n = 4$) over 48 h when injected intramuscularly at the hind limb.

5.2.1.2 Determination of minimum hemorrhagic dose (MHD)

Hemorrhagic activity was determined in mice, by a method modified from Theakston and Reid (1983) (in the Bulletin of the World Health Organization) where rats were used. The minimum hemorrhagic dose (MHD) was determined by injecting 40 μ L of varying amounts of venom (dissolved in normal saline) intradermally into the shaved dorsal skin of lightly sedated mice (with diethyl ether). After 90 min, the animals ($n = 3$ per dose) were killed by an overdose of diethyl ether and the dorsal skin was removed. The mean diameter of the hemorrhagic lesion on the inner surface of the skin was measured immediately upon skin removal (Figure 5.1a). Dose response curve between the mean diameter of the hemorrhagic lesion and venom dose was plotted. The MHD was the dose that caused a hemorrhagic lesion diameter of 10 mm.

5.2.1.3 Determination of minimum necrotic dose (MND)

As modified from Theakston and Reid (1983) (in the Bulletin of the World Health Organization), necrotic activity was determined by injection of the venom at varying amounts intradermally into the shaved dorsal skin of lightly sedated mice (with diethyl

ether), and the animals were given free access to water and feed *ad libitum*. After 72 h, the animals ($n = 3$ per dose) were killed by an overdose of diethyl ether and the dorsal skin was removed. The mean diameter of the necrotic lesion was then measured immediately upon skin removal (Figure 5.1b). The minimum necrotic dose (MND) is defined as the amount of venom that induces a necrotic lesion with a diameter of 5 mm.

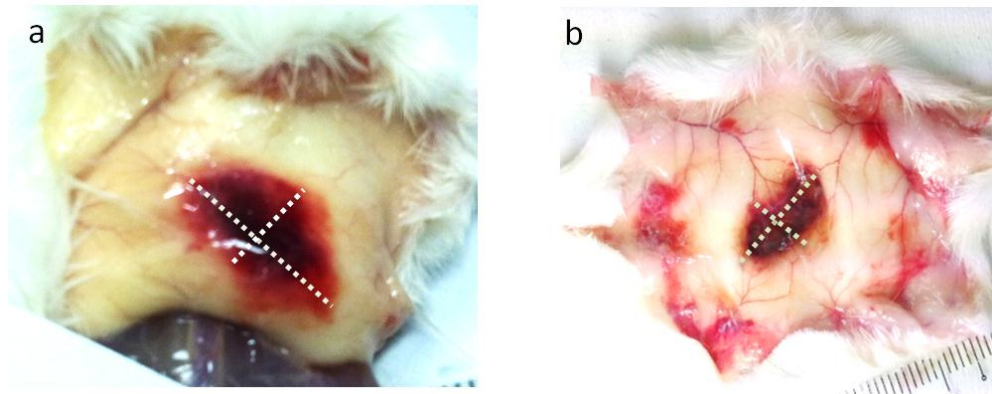


Figure 5.1 Measurement of the mean diameter for (a) a hemorrhagic spot, and (b) a necrotic spot. Mean diameter (mm) was determined from the average of the two widest diameters perpendicular to one another.

5.2.1.4 Determination of minimum coagulant dose (MCD)

Procoagulant activity was determined by the method adapted from Bogarín *et al.* (2000). Various amounts of venom, dissolved in 0.1 mL of normal saline, were added separately into 0.2 mL of bovine fibrinogen solution (2 g/L) or 0.2 mL of human citrated plasma at 37 °C. Coagulation times were recorded. The experiment was performed in triplicate. The minimum coagulant dose (MCD) is defined as the minimal amount of venom ($\mu\text{g/mL}$) that clots a standard solution of bovine fibrinogen or human citrated plasma in 60 s.

Using the methods described above, the lethal, hemorrhagic, necrotic and procoagulant doses of Malaysian *Calloselasma rhodostoma* venom were also established. Intravenous LD₅₀ of *Daboia russellii* (Sri Lanka) venom and *Echis carinatus sochureki* (Pakistan) venom were also determined in mice. These parameters from non-*H. hypnale* species were established for the purpose of comparing the neutralizing efficacy of selected antivenoms.

5.2.2 *In vitro* neutralization of the venom lethality, hemorrhagic, procoagulant and necrotic activities by antivenoms

These were carried out as modified from Ramos-Cerrillo *et al.* (2008). The antivenoms used were monovalent Malayan pit viper antivenom (MPVA) (Batch no. CR00909, exp.date 2/11/2014), Hemato polyvalent antivenom (HPA) (Batch no. HP00108, exp.date 6/11/2013) and Bharat Polyvalent ASVS (anti-snake venom serum) (Batch no.A5309049, exp.date 03/2013). The Thai antivenoms (Figure 5.2) were freeze-dried F(ab')₂ (90%) immunoglobulin fragments, obtained from hyperimmunized horses, refined by caprylic acid precipitation and pepsin digestion. The antivenoms were derived from equine plasma. For HPA, the horses were hyperimmunized with mixture of venoms from Malayan pit viper (*Calloselasma rhodostoma*), white-lipped green tree pit viper (*Cryptelytrops albolabris*) and Russell's viper (*Daboia simensis*), all of Thai origin. The Indian antivenom is a refined and concentrated preparation of F(ab')₂ obtained by fractionating antisera from hyperimmunized horses, and is capable of neutralizing cobra, common krait, Russell's viper and saw-scaled viper venoms according to the manufacturer (snake species not specified). The antivenoms were reconstituted with sterile water according to instructions and stored at -20 °C. All antivenoms were used before their expiry dates.



Figure 5.2 The monovalent Malayan pit viper antivenom (left) and Hemato polyvalent antivenom (right) produced by Thai Red Cross Society, Queen Saovabha Memorial Institute, Bangkok. The Hematopolyvalent antivenom is raised against venoms from Malayan pit viper (*Calloselasma rhodostoma*), white-lipped green tree pit viper (*Cryptelytrops albolabris*) and Russell's viper (*Daboia siamensis*), all of Thai origin. Both the antivenoms were gifts from the Queen Saovabha Memorial Insitute and used before the expiry dates. Each product contained ~1g freeze-dried $F(ab')_2$ immunoglobulin fragments, obtained from equine plasma, refined by caprylic acid precipitation and pepsin digestion.

5.2.2.1. Neutralization of lethality

Five times the intravenous median lethal dose of LD₅₀ of *H. hypnale* venom was mixed thoroughly with various dilutions of the antivenom in phosphate-buffered saline (PBS), to give a total volume of 200 μ L. The mixture was incubated at 37 °C for 30 min with gentle shaking. The mixture was subsequently centrifuged at 10,000 g and then injected intravenously into the caudal vein of mice (n = 4 per dose), and the number of animals that survived 48 h post-injection was recorded. Neutralization potency of the antivenom was measured by ED₅₀ (μ L antivenom/5 LD₅₀ venom), which is defined as the amount of antivenom (μ L) at which the survival ratio of the animals (challenged with the specified amount of venom) is 50%. The estimated amount of venom neutralized per mL of antivenom was then calculated based on the antivenom ED₅₀ value, the venom LD₅₀ value and body mass of animals used.

5.2.2.2 Neutralization of the hemorrhagic activity

Twice the minimum hemorrhagic dose (MHD) of *H. hypnale* venom was mixed thoroughly with various dilutions of the antivenom in PBS, to give a total volume of 40 μ L. The mixtures were incubated at 37 °C for 30 min with gentle shaking, before injected intradermally into the dorsal areas of lightly sedated mice (with diethyl ether) (n = 3 per dose). After 90 min, the animals were killed by an overdose of diethyl ether. The dorsal skin was removed immediately and the mean diameter of the hemorrhagic spot was measured. Neutralization potency of the antivenom was measured by ED₅₀ which is defined as the volume of antivenom (μ L) which reduced the activity of the challenge dose (2 MHDs) of the venom by 50%. For comparison purpose, it is also expressed in terms of the ratio of μ L antivenom per milligram venom.

5.2.2.3 Neutralization of necrotic activity

Two and a half times of minimal necrotic dose (MND) of *H. hypnale* venom (100 µg) was mixed well with various dilutions of the antivenoms in PBS to give a total volume of 50-80 µL. The mixtures were incubated at 37 °C for 30 min under gentle agitation, and subsequently injected intradermally into the dorsal areas of lightly sedated mice (with diethyl ether) (n = 3 per dose). The animals were given access to feed and water *ad libitum*. After 72 h, the animals were killed by an overdose of diethyl ether and following which, the skins were removed immediately to examine the mean diameter of the dermal necrotic lesion. Neutralization potency of the antivenom was measured by ED₅₀ which is defined as the volume of antivenom (µL) which reduced the necrotic activity of the challenge dose (2.5 MNDs) of the venom by 50%. For comparison purpose, it is also expressed in term of the ratio of µL antivenom per milligram venom.

5.2.2.4 Neutralization of procoagulant activity

Twice the minimum coagulant dose of *H. hypnale* venom were mixed thoroughly with various dilution of the antivenom in PBS, to give a total volume of 100 µL. The mixture was incubated at 37°C for 30 min with gentle shaking. To these, 200 µL of bovine fibrinogen (2 g/L) or human citrated plasma, pre-incubated at 37 °C, were added and the coagulation times were recorded. The experiment was repeated in triplicate. Neutralization potency of the antivenom was measured by effective dose (ED), which is defined as the volume of the antivenom (µL) at which coagulation time was increased three times when compared to the coagulation time of the fibrinogen solution or citrated human plasma incubated with venom alone. For comparison purpose, it is also expressed in terms of the ratio of µL antivenom per milligram venom.

The above neutralization assays were repeated for the lethal, hemorrhagic, necrotic and procoagulant effects of *C. rhodostoma* venom, using the Thai monovalent and polyvalent antivenoms. Neutralization of the lethal effect of *D. russelii* and *E. carinatus sochureki* venoms were tested using only the Thai polyvalent antivenom.

5.2.3 *In vivo* neutralization of the lethality of *Hypnale hypnale* venom by Hemato polyvalent antivenom using a rodent model

This was carried out by intramuscular injection of the minimum lethal dose (20 µg/g as predetermined) of *H. hypnale* venom into mice (n = 4) followed by intravenous injection of 200 µL of the Hemato polyvalent antivenom, 5 min after the venom inoculation. The number of animals survived 48 h post-injection was recorded. The local effects of the venom were also examined. The control group consisted of mice (n = 4) challenged with the same intramuscular minimum lethal dose of the venom, but followed by intravenous injection of 200 µL of normal saline.

5.3 RESULTS

5.3.1 Toxinological parameters of the venoms

The LD₅₀ of *H. hypnale* venom was determined as 0.90 µg/g (i.v.) and 13.7 µg/g (i.m.), as described in Chapter 4 (see 4.3.2). The i.v. LD₅₀ values of the venoms of *C. rhodostoma*, *D. russelii* and *E. carinatus sochureki* were, respectively, 1.48 µg/g (95% CI: 0.78-2.06 µg/g), 0.24 µg/g (95% CI: 0.19-0.62 µg/g) and 2.08 µg/g (95% CI: 1.02-4.42 µg/g).

H. hypnale venom exhibited strong procoagulant, hemorrhagic and necrotic activities. The minimum coagulation dose (MCD) is 56.2 µg/mL for bovine fibrinogen, and 55.1 µg/mL for human citrated plasma. The minimum hemorrhagic dose (MHD) and minimum necrotic dose (MND) are 10.5 µg and 39.3 µg, respectively. These values are comparable to those of the *C. rhodostoma* venom, with MCDs of 27.3 µg/mL for bovine fibrinogen and 24.9 µg/mL for human citrated plasma; MHD of 24.0 µg and MND of 28.7 µg, respectively.

These toxinological parameters were established for use in neutralization study, and are presented in Table 5.1.

5.3.2 *In vitro* neutralization of *Hypnale hypnale* and *Calloselasma rhodostoma* venoms

The abilities of three commercial antivenoms to neutralize the toxic activities of *H. hypnale* venom *in vitro* were examined. The neutralization was evaluated in assays

involving incubation of venom and antivenom for 30 min prior to testing on the various toxinological assays.

Bharat polyvalent antivenom (ASVS), the antivenom produced by immunizing horses with venoms from the 'Big 4' (*Naja naja*, *Bungarus caeruleus*, *Daboia russelii* and *Echis carinatus*) failed to protect against *H. hypnale* venom: all mice (n = 4) injected with 5 LD₅₀ (*i.v.*) of the venom died despite receiving 200 µL/mouse of the antivenom.

Table 5.1 and Table 5.2 showed the *in vitro* results of the monovalent Malayan pit viper (MPV) antivenom and Hemato polyvalent antivenom (HPA) in neutralizing the lethal, hemorrhagic, necrotic and procoagulant effects of *H. hypnale* venom. The neutralizing efficacies of the two antivenoms were studied simultaneously on the toxic effects of the venom of *C. rhodostoma* for comparison purpose. *C. rhodostoma* is regarded as the homologous species to which the antivenoms were produced.

Table 5.1 Neutralization of lethality of *Hypnale hypnale*, *Calloselasma rhodostoma* and other venoms by the monovalent Malayan pit viper antivenom (MPVA) and Hemato polyvalent antivenom (HPA).

Antivenom	Venom	LD ₅₀ (i.v.) µg/g	ED ₅₀ (µL antivenom / 5 i.v.LD ₅₀)	mg venom neutralized per mL antivenom
Monovalent Malayan pit viper antivenom	<i>C. rhodostoma</i>	1.48 µg/g (0.78-2.06)	41.53 µL (20.4-88.4)	3.23
	<i>H. hypnale</i>	0.90 µg/g (0.42-1.84)	70.71 µL (33.7-148.4)	0.89
Hemato polyvalent antivenom	<i>C. rhodostoma</i>	1.48 µg/g (0.78-2.06)	22.47 µL (14.8-34.1)	7.14
	<i>H. hypnale</i>	0.90 µg/g (0.42-1.84)	41.53 µL (20.4-88.4)	1.52
	<i>D. russelii</i>	0.24 µg/g (0.19-0.62)	7.52 µL (3.53-15.3)	2.50
	<i>E. carinatus</i> <i>sochureki</i>	2.08 µg/g (1.02-4.42)	> 200 µL	Not effective

Values in range for LD₅₀ and ED₅₀ indicated 95% confidence intervals. For neutralization experiments, mice (n = 4) were challenged with 5 i.v. LD₅₀ of the various venoms.

Table 5.2 Neutralization of the procoagulant, hemorrhagic and necrotic activities of *Hypnale hypnale* and *Calloselasma rhodostoma* venoms by the monovalent Malayan pit viper antivenom (MPVA) and Hemato polyvalent antivenom (HPA).

Toxic activity	Minimum dose	Neutralization by MPVA (ED or ED ₅₀)	Neutralization by HPA (ED or ED ₅₀)
Procoagulant	MCD		
<i>H. hypnale</i>	56.2 ± 1.3 µg/mL [*]	ED = 4.8 ± 0.1 µL [*] (432.1 ± 10.9 µL/mg)	ED = 1.3 ± 0.0 µL [*] (114.2 ± 2.7 µL/mg)
	55.1 ± 1.4 µg/mL [#]	ED = 4.2 ± 0.1 µL [#] (384.4 ± 11.2 µL/mg)	ED = 1.3 ± 0.0 µL [#] (121.3 ± 0.7 µL/mg)
<i>C. rhodostoma</i>	27.3 ± 0.3 µg/mL [*]	ED = 1.1 ± 0.0 µL [*] (209.2 ± 0.2 µL/mg)	ED = 0.7 ± 0.0 µL [*] (133.9 ± 0.3 µL/mg)
	24.9 ± 0.4 µg/mL [#]	ED = 0.8 ± 0.0 µL [#] (152.7 ± 3.7 µL/mg)	ED = 0.5 ± 0.0 µL [#] (104.0 ± 1.2 µL/mg)
Hemorrhagic	MHD		
<i>H. hypnale</i>	10.5 ± 0.6 µg	ED ₅₀ = 9.9 ± 0.3 µL (472.3 ± 13.9 µL/mg)	ED ₅₀ = 1.4 ± 0.1 µL (67.4 ± 5.1 µL/mg)
<i>C. rhodostoma</i>	24.0 ± 0.9 µg	ED ₅₀ = 7.3 ± 1.4 µL (151.7 ± 29.3 µL/mg)	ED ₅₀ = 5.9 ± 0.2 µL (122.8 ± 3.9 µL/mg)
Necrotic	MND		
<i>H. hypnale</i>	39.3 ± 1.6 µg	ED ₅₀ = 61.2 ± 1.7 µL (612.6 ± 17.5 µL/mg)	ED ₅₀ = 5.3 ± 0.1 µL (53.8 ± 0.59 µL/mg)
<i>C. rhodostoma</i>	28.7 ± 2.6 µg	ED ₅₀ = 2.7 ± 0.1 µL (38.1 ± 1.9 µL/mg)	ED ₅₀ = 2.1 ± 0.0 µL (29.7 ± 0.4 µL/mg)

Neutralization of hemorrhagic and procoagulant activities were determined by challenge with 2 MHDs and incubation with 2 MCDs, respectively, of the venom. For neutralization of necrotic activity, mice were challenged with 2.5 MNDs of venom intradermally. Values are expressed as mean ± S.E.M. (n= 3 for procoagulant, hemorrhagic and necrotic activities). The standard volume of antivenom was expressed in µL and the amount of venom neutralized was in mg.

^{*} Procoagulant activity tested on bovine fibrinogen

[#] Procoagulant activity tested on human citrated plasma

5.3.3 *In vitro* neutralization of the lethalities of *Daboia russelii* and *Echis carinatus sochureki* venom by Hemato polyvalent antivenom

The ability of Hemato polyvalent antivenom to neutralize the lethal effects of two common vipers (*D. russelii* and *E. carinatus*) found in Sri Lanka and India subcontinent was also investigated. The *in vitro* results of the neutralization assay in mice were included in Table 5.1.

5.3.4 *In vivo* neutralization of *Hypnale hypnale* venom in a rodent model of envenomation

To further evaluate the potential of the application of Hemato polyvalent antivenom in the treatment of *H. hypnale* bites, an *in vivo* neutralization experiment, using a rodent model was carried out. In this experiment, four mice received intravenous injection of 200 µL of Hemato polyvalent antivenom 5 min after *i.m.* injection of minimum lethal dose (400 µg per mouse) of *H. hypnale* venom. All four mice survived, and observations of the treated animals indicated that in addition to *in vivo* neutralization of the venom lethality, the antivenom also prevented or largely reduced the occurrence of venom-induced local tissue damage (myonecrosis and bleeding from the injection site) of *H. hypnale* venom.

5.4 DISCUSSION

5.4.1 *In vitro* neutralization of the toxinological activities of snake venoms

5.4.1.1 Against *Hypnale hypnale* and *Calloselasma rhodostoma* venoms

Envenomation by *H. hypnale* receives much attention in recent years owing to re-identification of the biting snake and subsequent authentication of its high prevalence with severe clinical outcomes. To date, *H. hypnale* envenoming remains an endemic medical crisis in the absence of an effective antivenom available in the region. Bharat polyvalent antivenom used in the region for treating the Big Four envenomation (Joseph *et al.*, 2007) is totally not effective against Sri Lankan *H. hypnale* venom in the current cross-neutralization study using a rodent model. This is consistent with the clinical observations reported by Joseph *et al.* (2007) that administration of Bharat polyvalent antivenom did not bring any benefits to patients envenomated by *H. hypnale* in the Southwestern coast of India. Other authors also reported that the Haffkine polyvalent antivenom (also produced against the ‘Big Four’) was totally ineffective in the treatment of victims bitten by *H. hypnale* (Sellahewa *et al.*, 1995; Ariaratnam *et al.*, 2008), highlighting the distinct uniqueness of *H. hypnale* venom antigenic profiles from the Big Four’s, hence needing a more specific or paraspecific antivenom for effective treatment.

The capability of the monovalent Malayan pit viper antivenom (MPVA) to neutralize *H. hypnale* venom was then examined, as Malayan pit viper (*C. rhodostoma*) is considered a sister taxon to *H. hypnale* by mitochondrial DNA analysis (Parkinson *et al.*, 1997). Ariaratnam *et al.* (2008) reported that (expired) MPVA failed to neutralize 5 *i.v.*LD₅₀ of *H. hypnale* venom *in vitro*, even with 200 µL of the antivenom. A

preliminary study, however, showed that when lesser amount of *H. hypnale* venom were used (2 LD₅₀, intraperitoneal injection), the newly manufactured MPVA could neutralize the lethality of the venom in mice, though moderately. The present study showed that the newly manufactured MPVA indeed could effectively protect mice injected with 5 *i.v.* LD₅₀ of *H. hypnale* venom, and the ED₅₀ was determined to be 70.71 µL/5 *i.v.* LD₅₀, or equivalent of 0.89 mg venom neutralized per mL of antivenom. The discrepancies with the previous report by Ariaratnam *et al.* (2008) could be due to batch differences in the antivenom, as the current batch of the antivenom used in this study was manufactured using a new process (caprylic acid precipitation) and appears to be more potent (Khomvilai, 2008). The MPVA was also effective in the neutralization of the procoagulant effect on both bovine fibrinogen and human citrated plasma, with EDs of 432.1 µL/mg venom and 384.4 µL/mg venom; respectively. It also neutralized the hemorrhagic and necrotic activities of *H. hypnale* venom, with ED₅₀s of 472.3 µL/mg venom and 612.6 µL/mg venom, respectively. As expected, the MPVA was much more potent in the neutralization of the lethality and toxicities ($p < 0.05$) of the homologous *C. rhodostoma* venom, in particular the necrotic and hemorrhagic activities. The ED₅₀ against *C. rhodostoma* venom lethal effect was 41.53 µL/5 *i.v.* LD₅₀, or equivalent of 3.23 mg venom neutralized per mL of antivenom. The neutralization ED/ED₅₀s are 209.2 µL/mg and 152.7 µL/mg for procoagulant activities against bovine fibrinogen and human citrated plasma, respectively; 151.7 µL/mg for hemorrhagic activity and 38.1 µL/mg for necrotic activity of the venom. Nevertheless, these results suggest that at least some of the venom toxins from the two crotalids, *H. hypnale* and *C. rhodostoma*, are antigenically similar. It is known that the procoagulant enzymes (thrombin-like enzymes) and hemorrhagins from different venomous snakes can have very different antigenic properties. For example, thrombin-like enzyme from *Cryptelytrops purpureomaculatus* exhibited very little ELISA cross reactions with the thrombin-like

enzymes from *C. rhodostoma* venom (Tan, 2010). Also, Fung (2002) reported that the major hemorrhagin of *C. purpureomaculatus* venom did not cross-react with *C. rhodostoma* venom at all when examined on double-sandwich ELISA. The distinct antigenic differences may be associated with variation in niche and diet, as it is noted that both *C. rhodostoma* and *H. hypnale* are mainly terrestrial while *C. purpureomaculatus* is an arboreal species. The ability of the MPVA to neutralize the lethality, procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom therefore also supports the conclusion by Parkinson et al (1997) that *C. rhodostoma* is a sister taxon to *H. hypnale*. This is an interesting example of similarities in the immunological properties of the venom proteins support phylogenetic relationship derived from mitochondrial DNA analysis. The close phylogenetic relationship between *H. hypnale*, found in India subcontinent and *C. rhodostoma*, found in Southeast Asia, is a fascinating example of the existence of the ‘Malayan’ element in the fauna of Peninsular India. This phenomenon has been known since a long time, and the cause of these similarities have been explained by the Satpura Hypothesis (Hora, 1949), according to which fauna migration followed the Satpura trends of mountain through Peninsular India during the Pleistocene. Karanth (2003) provided an alternative hypothesis that the phenomenon was instead a result of convergent evolution.

Recently, a new polyvalent antivenom against hematoxic snake venoms in Thailand has become available. This polyvalent antivenom, termed Hemato polyvalent antivenom, was produced from plasma of horses hyperimmunized by venoms from the three common viper and pit vipers in Thailand: Green pit viper (*Cryptelytrops albolabris*), Russell’s viper (*Daboia siamensis*) and Malayan pit viper (*C. rhodostoma*). The Hemato polyvalent antivenom was found to be much more effective than the MPVA in neutralizing the lethality, procoagulant, hemorrhagic and necrotic activities of *C.*

rhodostoma venom: the ED₅₀ for neutralization of the lethality was determined to be 22.47 µL/5 i.v. LD₅₀, or 7.14 mg venom neutralized per mL of antivenom. The EDs for the neutralization of procoagulant activities against bovine fibrinogen and human citrated plasma are 133.9 µL/mg and 104.0 µL/mg venom, correspondingly; whereas the ED₅₀s for the neutralization of hemorrhagic and necrotic activities are 122.8 µL/mg venom and 29.7 µL/mg venom respectively. It is therefore not surprising to find that the Hemato polyvalent antivenom is also very effective in the neutralization of the lethality, procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom. Against 5 LD₅₀ (i.v.), the ED₅₀ of the polyvalent antivenom was determined to be 41.53 µL, which is equivalent to neutralization of 1.52 mg of *H. hypnale* venom per mL of the reconstituted Hemato polyvalent antivenom. In comparison, 1 mL of MPVA neutralized only 0.89 mg of the venom. Comparison of the ED/ED₅₀s of neutralization of procoagulant, hemorrhagic and necrotic activities of the venom also showed that the Hemato polyvalent antivenom is far more effective ($p < 0.05$) than the MPVA in neutralizing the toxic activities of the *H. hypnale* venom. In fact, the ED₅₀s of the neutralization of the procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom by the Hemato polyvalent antivenom is comparable to the ED/ED₅₀s against *C. rhodostoma* venom although the antivenom is more effective against *C. rhodostoma* than *H. hypnale* in term of neutralization of lethality (7.14 mg venom/mL antivenom, versus 1.52 mg venom/mL antivenom, $p < 0.05$).

The greater efficacy of the Hemato polyvalent antivenom than the MPVA in the neutralization of *H. hypnale* (as well as *C. rhodostoma*) venom suggested that the inclusion of other two venoms (of *D. siamensis* and *C. albolabris*) used in the immunization scheme of preparation of the polyvalent antivenom could have synergistically enhanced the immunogenicity and the antivenom production. The

venoms may also contain venom toxins that are immunologically similar to some *H. hypnale* venom toxins, resulting in the production of some cross reacting antibody. The current preclinical studies therefore suggest that both the monovalent Malayan pit viper and Hemato polyvalent antivenom may be useful in the antivenom treatment of systemic *H. hypnale* envenoming. The polyvalent antivenom might be the preferred one because of its greater efficacy, though the drawback is its higher cost (USD 60 per vial compared to USD 40 per vial for the monovalent antivenom). In view of the relatively low yield of venom from *H. hypnale* (average of 13 mg per milking, Section 4.3.1) because of its relatively small size, and the strong neutralization capacity of the Hemato polyvalent antivenom, 1-2 vials (10-20 mL reconstituted antivenom) of the polyvalent antivenom would probably be sufficient in the treatment of most systemic *H. hypnale* envenomation. Nevertheless, while the results of the preclinical studies are promising, these studies must be followed by a randomized controlled clinical trial in the relevant regions, which is the final criterion for assessing the clinical efficacy and safety of the antivenom.

5.4.1.2 Against *Daboia russelii* and *Echis carinatus* venoms

Daboia russelii and *Echis carinatus* are two vipers that contribute significantly to snakebite envenomation in the region. Unfortunately, it is not always possible to identify the biting species among these vipers and *H. hypnale*. The cross-neutralization study however showed that the Hemato polyvalent antivenom could neutralize the Sri Lankan *D. russelii* venom effectively, with an ED₅₀ of 7.52 µL/5 LD₅₀ (i.v.), or equivalent to 2.50 mg venom per mL antivenom. The Hemato polyvalent antivenom indeed was raised against a mixture of 3 venoms, including the Thai *D. siamensis* venom, which presumably contains some common antigens with the Sri Lanka *D.*

russelii venom to which cross-neutralization occurs. The polyvalent antivenom, however, was not effective in the neutralization of *E. carinatus sochureki* venom: all 4 mice that were injected with 5 LD₅₀ (*i.v.*) died despite receiving a maximum of 200 µL of the antivenom. When the cross-neutralization assay was performed, the *E. carinatus* venom used was sourced from a subspecies *sochureki* found in Pakistan and Northern India, and not exactly the subspecies *sinhaleyus* endemic to Sri Lanka, due to the unavailability of Sri Lankan specimen and its venom at that time of study. Despite the difference in subspecies status, we believed that in general the toxin antigenic profile of *E. carinatus* venom is far different from the three venoms (of *C. rhodostoma*, *D. siamensis* and *C. albolabris*) used in the production of Hemato polyvalent antivenom, as indicated by the total ineffectiveness of the polyvalent antivenom against it. Nevertheless, the ability of the Hemato polyvalent antivenom to neutralize *D. russelii* venom increases the potential benefit of the use of Hemato polyvalent antivenom in management of viper/pit viper bites in Sri Lanka and Southern India, as *D. russelii* is the second commonest cause of snake bite in the region, next to *H. hypnale* (Ariaratnam *et al.*, 2009). Since clinically it is not always possible to distinguish between envenomation by *H. hypnale*, *D. russelii* and *E. carinatus*, double-sandwich ELISA should be developed to assist in biting species identification in future clinical trials of the antivenoms.

5.4.2 *In vivo* neutralization of the lethality of *Hypnale hypnale* venom

The Hemato polyvalent antivenom has been specifically used in the *in vivo* neutralization assay in view of its greater *in vitro* efficacy and potentially better cost-effectiveness compared to that of the monovalent antivenom. It was noted in this study that mice challenged by the venom died much faster than what was reported in human

cases (Ariaratnam *et al.*, 2008), hence “5 minutes post-envenomation” has been standardized as the point to begin antivenom injection. This was done essentially to validate the *in vivo* effectiveness of the antivenom, although the time of intervention might not be reflective of actual clinical situation and hence may require extended time points in future study. Nevertheless, the prevention of death and attenuation of local tissue damages conferred by Hemato polyvalent antivenom *in vivo* is indeed consistent with the result of *in vitro* neutralization of the lethal, necrotic and hemorrhagic activities of *H. hypnale* venom. Immunotherapy to abrogate venom-induced local tissue destruction represents a difficult challenge, and neutralization was shown to achieve only to a partial extent in several studies (Léon *et al.*, 1997; Léon *et al.*, 2000; Gutiérrez *et al.*, 2003). The neutralization of local effects, however, though may not appears very promising, is nonetheless possible and potentially useful in viper and pit viper bites where local microvasculature changes (i.e. increase in vascular permeability and hemorrhage) favor the extravasation of antivenom, leading to local neutralization (Léon *et al.*, 2001). The *in vivo* result in this study is qualitatively consistent with this concept and the observations reported previously.

The cross-neutralization results (*in vitro* and *in vivo*) indicated that there are substantial immunological cross-reactivities between toxic components present in *H. hypnale* venom and the viperid (most notably *C. rhodostoma*) venoms used in the production of the Thai antivenoms, despite differences in the species. Extensive cross-neutralization between several *Bothrops* venoms and antivenoms has been described (Bogarín *et al.*, 2000; Rojas *et al.*, 2005). The results therefore support the hypothesis that some antivenoms can be effective against heterologous venoms in other countries and may be helpful in situations where locally produced antivenoms are not available, as demonstrated by Otero *et al.* (1996).

CHAPTER 6

Nephrotoxicity of *Hypnale hypnale* Venom and Neutralization

6.1 INTRODUCTION

Kidney, as a highly vascularized excretory organ, is certainly vulnerable to toxic effects of venoms, especially from vipers and pit vipers, venoms of which have been conveniently categorized as ‘hematoxic’ and/or ‘myotoxic’ type. In envenomation, toxin enzymes like phospholipases A₂ and various proteases can initiate inflammatory response that generates proinflammatory cytokines and vasoactive mediators, resulting in systemic and renal hemodynamic alterations with decreased renal blood flow. Toxin enzymes also exert direct effects on erythrocytes, myocytes, blood coagulation factors (including fibrinogen), and vascular endothelial cells, leading to intravascular hemolysis, rhabdomyolysis, consumptive coagulopathy, bleeding diathesis, etc. that further compromise renal perfusion. In some instances, direct tubular nephrotoxicity is exhibited by venoms with strong cytolytic enzymes that target the renal tubular epithelial cells. Kidneys respond to venom effects at various degrees, and the manifestation is often acute kidney injury (AKI), attributed to the combined effects of reduced renal blood flow and consumptive coagulopathy or direct tubular toxicity (Kanjanaabuch and Sitprija, 2008; Sitprija, 2008). In a series of over 300 hump-nosed pit viper bites, Ariaratnam *et al.* (2008) reported that 117 (39%) were systemically envenomed, characterized by hemostatic abnormalities. Thirty patients out of these 117 cases developed acute renal failure, suggesting some associations between the two pathologies. The renal complication resulted in death or chronic morbidity (chronic kidney disease); hence it is a relevant toxic feature to be studied and, more importantly, tested for neutralization by potential antivenoms.

6.2 METHODS

6.2.1 Determination of venom median lethal dose (LD₅₀) for nephrotoxicity study

The intramuscular and intraperitoneal LD₅₀ values of *H. hypnale* venom were respectively standardized at 13.7 µg/g and 6.0 µg/g as determined in a mouse model, described in Chapter 4 (see 4.3.2).

The intramuscular LD₅₀ value of *Daboia siamensis* (Myanmar origin) venom was determined by injecting 100 µL venom in PBS into the quadriceps muscles of mice (n = 4 per dose), whose survival ratio was recorded after 48 hours. The LD₅₀ (and the 95% confidence intervals, CI) of the venom was calculated using the Probit analysis method of Finney (1952). The venom of *Daboia siamensis* was used as a positive control for inducing acute kidney injury (see 6.4.1.1)

6.2.2 Preliminary study of *in vivo* nephrotoxic effect of *Hypnale hypnale* venom at a sublethal dose in rats

The intramuscular LD₅₀ for Sprague Dawley rats were adapted from the values determined in mice as described. Sprague Dawley rats were divided into three groups of three animals each: normal saline (NS) group, *H. hypnale* venom (HV) group and *Daboia siamensis* (Myanmar) venom (DV) group. The NS group and DV groups represented, respectively, the negative and positive controls. Rats from each group received intramuscular injections of treatment as follows: 200 µL of normal saline or 200 µL of venom solution corresponding to one third of the venom's intramuscular LD₅₀ (predetermined to be sublethal). The rats were individually housed in metabolic

cage with access to food and water *ad libitum* for 24 hours. Urine was collected throughout 24 hours, while blood was collected via cardiac puncture under anesthesia with a mixture of xylazine and ketamine (1 and 10 mg, respectively, per 200 g rat) at the end of experiment. The urine and blood samples were sent for biochemical analysis, to an outsourced service at a pathology analytical laboratory (Pathology & Clinical Laboratory, Malaysia). Subsequently, the rats were euthanized by cervical dislocation, and the major organs harvested from rats were sent for histopathological studies. The organ specimens were fixed in 10% formalin in PBS, followed by paraffin wax-embedding, sectioning of tissue, dewaxing, rehydration and staining with hematoxylin and eosin. Wax-embedding, sectioning and staining of tissues were conducted at the Pathology's Unit for Research and Education (Department of Pathology, University of Malaya). The sections were cover-slipped and images were captured using a Nikon Research Trinocular Teaching Microscope Eclipse 80i® equipped with a camera (Japan).

6.2.3 Nephrotoxicity study of *Hypnale hypnale* venom at lethal doses in mice

Further study on the venom nephrotoxicity involved the use of albino mice of ICR-strain (instead of rats) and intraperitoneal route of envenomation (instead of intramuscular route) to minimize the amount of venom used in view of the explorative nature of the study. Albino mice of ICR-strain in this study were divided into one control group and two challenge groups (Group A and Group B). Mice in the control group (n = 4) each received 200 µL normal saline; while mice in Group A (n = 9) each received *H. hypnale* venom at 1 intraperitoneal LD₅₀ (6 µg/g), and those in Group B (n = 4) each received the same venom at 1.5 intraperitoneal LD₅₀ (9 µg/g). The venom was dissolved in normal saline and the volume of injection was adjusted to 200 µL. Venom

and vehicle (normal saline) were all injected intraperitoneally. Upon receiving the injections, mice were housed individually in standard metabolic cages with access to food and water *ad libitum*. Hourly observations on the mice's behaviors and evidence of toxic signs were charted over 48 hours. Mice that showed features suggestive of impending death (labored breathing with pauses, feeble heartbeat, loss of righting reflex, inability to walk, and lack of response to manipulation) were more closely monitored until spontaneous death, upon which blood was collected immediately from the heart, and this was followed by dissection to harvest major organs for gross and microscopic examinations.

At 48 h, all surviving mice were sedated with a mixture of xylazine and ketamine (0.1 and 1.0 mg, respectively, per 20 g mouse) and blood was collected via cardiac puncture. Subsequently, the mice were euthanized by cervical dislocation, and the major tissues/organs were harvested. Blood collected in plain tubes were observed for clotting time before processing for blood urea and creatinine analysis. Urine collected throughout the study period was tested on urine test strips for specific gravity, hematuria and proteinuria. The major organs harvested upon death were fixed in 10% formalin in PBS, followed by paraffin wax-embedding, sectioning of tissue, dewaxing, rehydration, and staining with hematoxylin and eosin. Kidney tissues were specifically sectioned at 1.5 μ m thin layer.

6.2.4 Neutralization of nephrotoxicity of *Hypnale hypnale* venom by antivenom

The experimental envenoming with 1 LD₅₀ and 1.5 LD₅₀ doses was repeated in Group C (n = 9) and Group D (n = 4), respectively. Intervention was carried out 5 minutes following the venom challenge, where each mouse from both groups received an

injection of 200 μ L of Hemato polyvalent antivenom (reconstituted as 1 vial of HPA: 10 ml normal saline) via the caudal vein. Animal housing, monitoring of signs, collection of blood and urine samples, processing of tissues/organs of the mice and further analyses were performed essentially as described in the nephrotoxic model described in Section 6.2.3. The significance of the differences of the means of the results was determined by one-way ANOVA and Tukey's post hoc test.

6.2.5 A preliminary study on time-dependent effect of *Hypnale hypnale* venom on mouse renal function and histology

Twenty albino ICR mice were intraperitoneally injected with 1 LD₅₀ of *H. hypnale* venom, and individually housed in metabolic cage with free access to food and water, similar to that described in nephrotoxic modelling (see 6.2.3). Mice that survived envenomation (beyond day 2, n = 12) were sacrificed at specific day interval (day 5, 10, 15 and 21; n = 3 at each interval). Sedation, euthanasia, collection of urine (last 24 hours) and blood, processing of tissues/organs of the mice and further analyses were performed essentially as described in 6.2.3. The significance of the differences of the means of the results was determined by one-way ANOVA and Tukey's post hoc test.

6.3 RESULTS

6.3.1 Intramuscular median lethal dose (LD₅₀) of *Daboia siamensis*

The LD₅₀ value of *D. siamensis* (Myanmar origin) was determined to be 0.70 µg/g (95% CI: 0.45-0.90 µg/g) in mice.

6.3.2 Preliminary nephrotoxicity study of *Hypnale hypnale* venom at sublethal dose in rats

Rats from both HV and DV groups treated with sublethal dose (1/3 LD₅₀) of venoms of *H. hypnale* and *D. siamensis* respectively, exhibited envenoming signs e.g. local hemorrhage, malaise, drowsiness and limping in the first few hours. All animals survived through 24 hours observation. Gross inspection on urine samples showed marked hematuria for the DV group, with no difference in the urine volumes among the groups. Urine biochemical and microscopic analysis revealed significant proteinuria and hematuria in the DV group compared to the HV and NS groups ($p < 0.05$) (Table 6.1). The blood urea and creatinine levels, used as indicators for acute kidney injury, were observably higher in the DV group although not significantly different from the other two groups (Table 6.1). Histological examination of the organs revealed no major differences between rats from the HV group and NS control group (Figure 6.1).

Table 6.1 Effects of sublethal dose (1/3 intramuscular LD₅₀) of *Hypnale hypnale* and *Daboia siamensis* venoms on blood and urine parameters (n = 3).

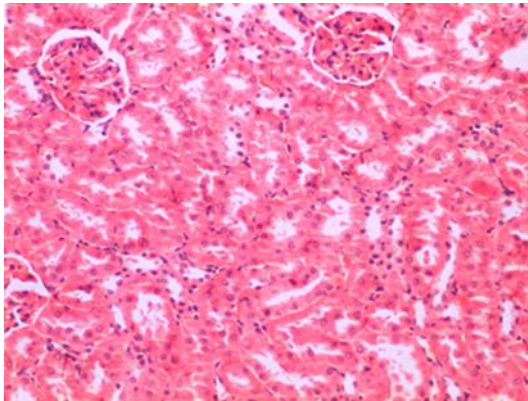
Parameter (mean ± SEM)	Group of rat Normal saline	<i>Hypnale hypnale</i> venom	<i>Daboia</i> <i>siamensis</i> venom
<i>Urine</i>			
Color	Straw	Straw	Reddish
Appearance	Clear	Clear	Hazy
Volume (ml)	4.2 ± 0.7	4.0 ± 0.8	3.9 ± 0.7
Protein (+)	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.5*
Blood (Hematuria) (+)	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.8*
Microscopic red blood cell	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 0.9*
Nitrite (+)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Bacteria (+)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
<i>Blood</i>			
Urea (mmol/l)	6.0 ± 0.8	6.9 ± 0.6	14.5 ± 7.0
Creatinine (μmol/l)	18.7 ± 3.2	18.0 ± 3.6	32 ± 13.0

* Significantly different from the other two groups, $p < 0.05$, one-way ANOVA

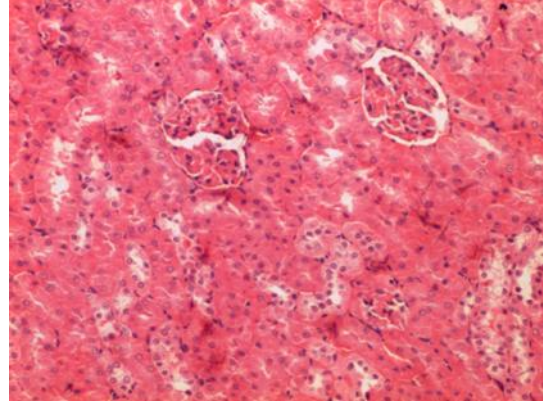
(Figure 6.1)

a) Kidney

a(i)

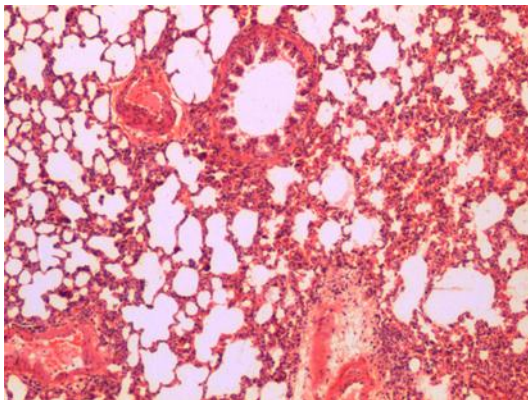


a(ii)

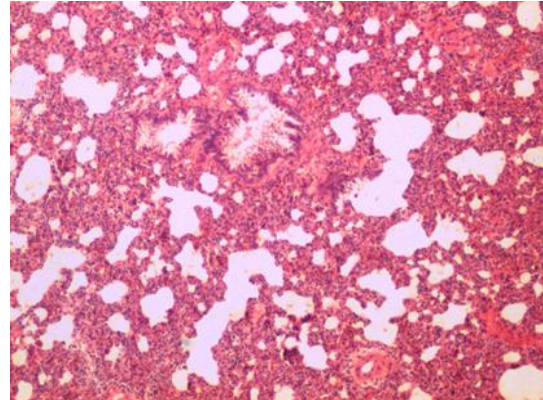


b) Lung

b(i)

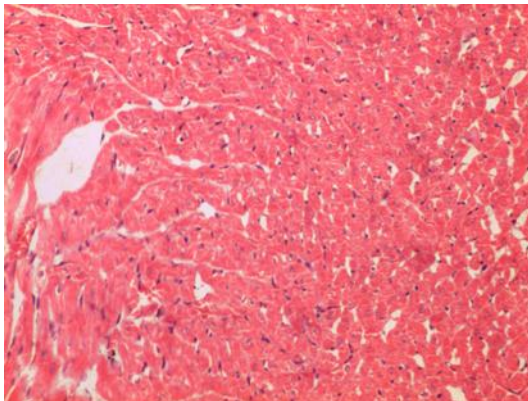


b(ii)

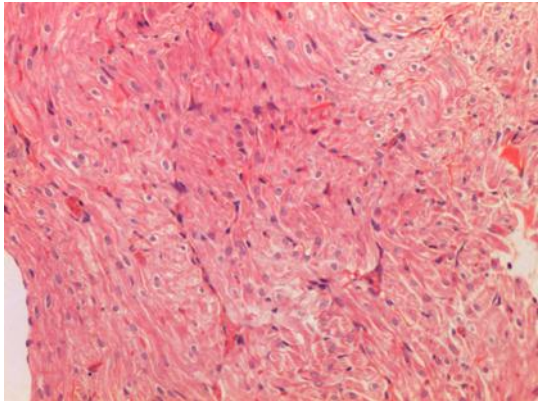


c) Heart

c(i)



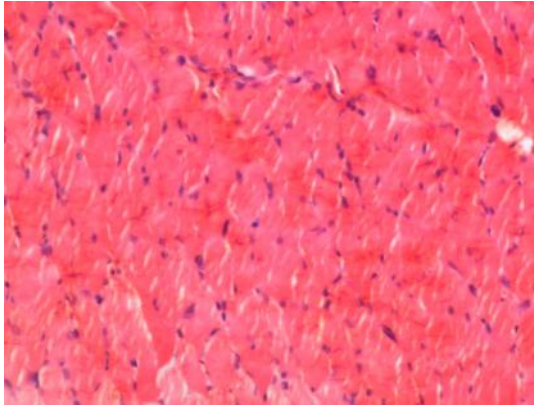
c(ii)



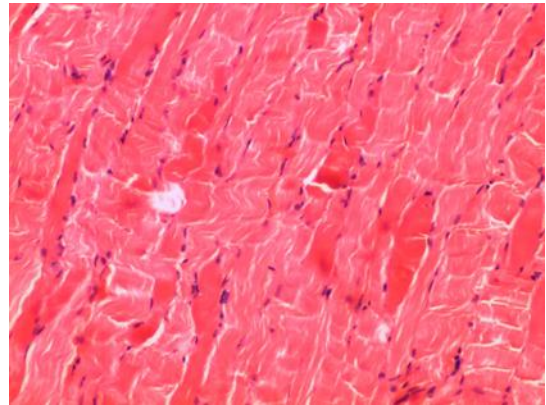
(Figure 6.1, continued.)

d) Diaphragm

d(i)

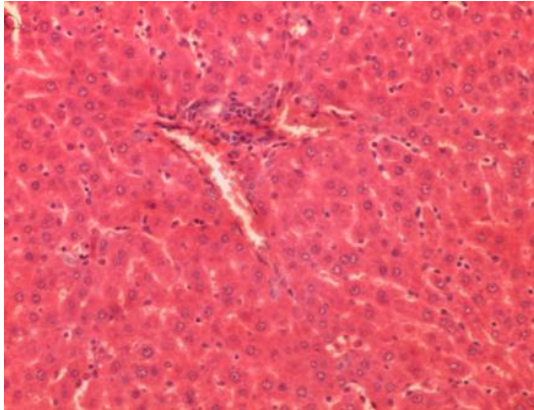


d(ii)

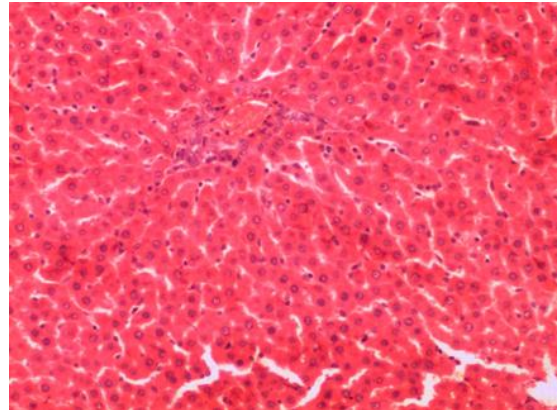


e) Liver

e(i)

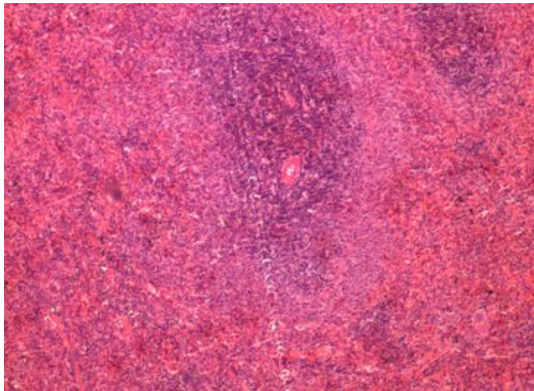


e(ii)



f) Spleen

f(i)



f(ii)

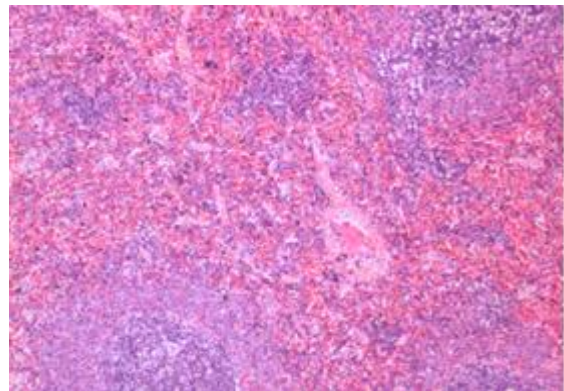


Figure 6.1 Histological findings of the effect of *Hypnale hypnale* venom on major organ-tissues of rat. Representative formalin-fixed, paraffin-embedded tissue sections of a(i) kidney of HV group, a(ii) kidney of NS group, x100; b(i) lung of HV group, b(ii) lung of NS group, x40; c(i) heart of HV group, c(ii) heart of NS group, x100; d(i) diaphragm of HV group, d(ii) diaphragm of NS group, x100; e(i) liver of HV group, d(ii) liver of NS group, portal track, x100; f(i) spleen of HV group, f(ii) spleen of NS group, x40.

HV group: Challenge group (rats injected intramuscularly with *H. hypnale* venom at 1/3 LD₅₀)

NS group: Control group (rats received normal saline intramuscularly as control)

6.3.3 Nephrotoxicity study of *Hypnale hypnale* venom at lethal doses in mice

Hypnale hypnale venom at median lethal dose (LD₅₀) killed 5 out of 9 mice (Group A) (fatality rate: 56%) within the observatory period. The blood urea and creatinine levels were noticeably increased (Figure 6.2a), and the clotting time was prolonged (Table 6.2). Hematuria and proteinuria were also present in all mice experimentally envenomed with the venom (Table 6.2).

In mice challenged with 1.5 LD₅₀ (Group B), severe azotemia (with blood creatinine level increased much higher than urea level) was observed in all mice (Figure 6.2b). The blood clotting time was prolonged, with significant proteinuria in the absence of hematuria detected (Table 6.2). On autopsy, patches of pallor were noted on the renal cortices of the challenged mice (Figure 6.3a). However, histopathological examination did not reveal remarkable findings under light microscopy within the study period (48 h maximum) for most of the challenged mice (> 80%). In minority (one each from Group A and B), there was mild degree of tubular dilation, vascular congestion and epithelial denudation of the kidneys (Figure 6.4a(i) & b(i)-(ii)), and hemorrhage in the lungs (Figure 6.3b; Figure 6.4c(i)). Table 6.2 also showed that the clotting time was prolonged, and the urine specific gravity was marginally raised in the challenged mice ($p = 0.04$).

Table 6.2 Effects of *Hypnale hypnale* venom on urine and blood parameters of mice experimentally envenomed with 1 LD₅₀ (Group A) and 1.5 LD₅₀ (Group B) venom.

Test Group	Time to death (h)	Urine			Blood		
		Specific gravity	Hematuria (+)	Proteinuria (+)	Clotting time (s)	Urea (mmol/L)	Creatinine (μmol/L)
Control (n = 4)	Not observed	1.023 ± 0.002	0.0 ± 0.0	1.0 ± 0.0	12.5 ± 2.9	7.8 ± 0.6	19.3 ± 3.1
A (n = 9)	18.4 ± 2.1	1.026 ± 0.001	1.8 ± 0.6 ^a	1.9 ± 0.3	28.9 ± 6.5 ^b	19.0 ± 4.5	28.6 ± 7.6
B (n = 4)	7.4 ± 1.3	1.029 ± 0.001 ^a	0.0 ± 0.0 ^a	1.8 ± 0.3	45.0 ± 2.9 ^b	14.4 ± 1.0	76.3 ± 3.3 ^c

Venom was dissolved in normal saline and volume was adjusted to 200 uL for intraperitoneal injection. Data were expressed as means ± SEM. Fatality in Group A = 56%; Group B = 100%.

Means (for Groups A and B) indicated with letters showed significant difference from the corresponding means of control for the test, as determined by one-way ANOVA and Tukey's post hoc test: ^a $p \leq 0.05$; ^b $p \leq 0.01$; ^c $p \leq 0.001$.

6.3.4 Neutralization of nephrotoxicity of *Hypnale hypnale* venom with antivenom

Death and nephrotoxicity (AKI as a likely cause of death) were effectively prevented by Hemato polyvalent antivenom (HPA) in this *in vivo* rodent model, evidenced by the normalization of blood and urine biochemistry of the envenomed animals (Figure 6.2a: significant amelioration of hematuria ($p \leq 0.001$), proteinuria ($p \leq 0.001$) and ureamia ($p \leq 0.05$); Figure 6.2b: significant amelioration of proteinuria ($p \leq 0.05$), ureamia ($p \leq 0.001$) and azotemia ($p \leq 0.001$)). Moreover, in the intervened groups, urine specific gravity improved significantly ($p \leq 0.05$), along with normal anatomy of the kidneys and lungs (Figure 6.3a-b and 6.4a-b) as well as 100% survival.

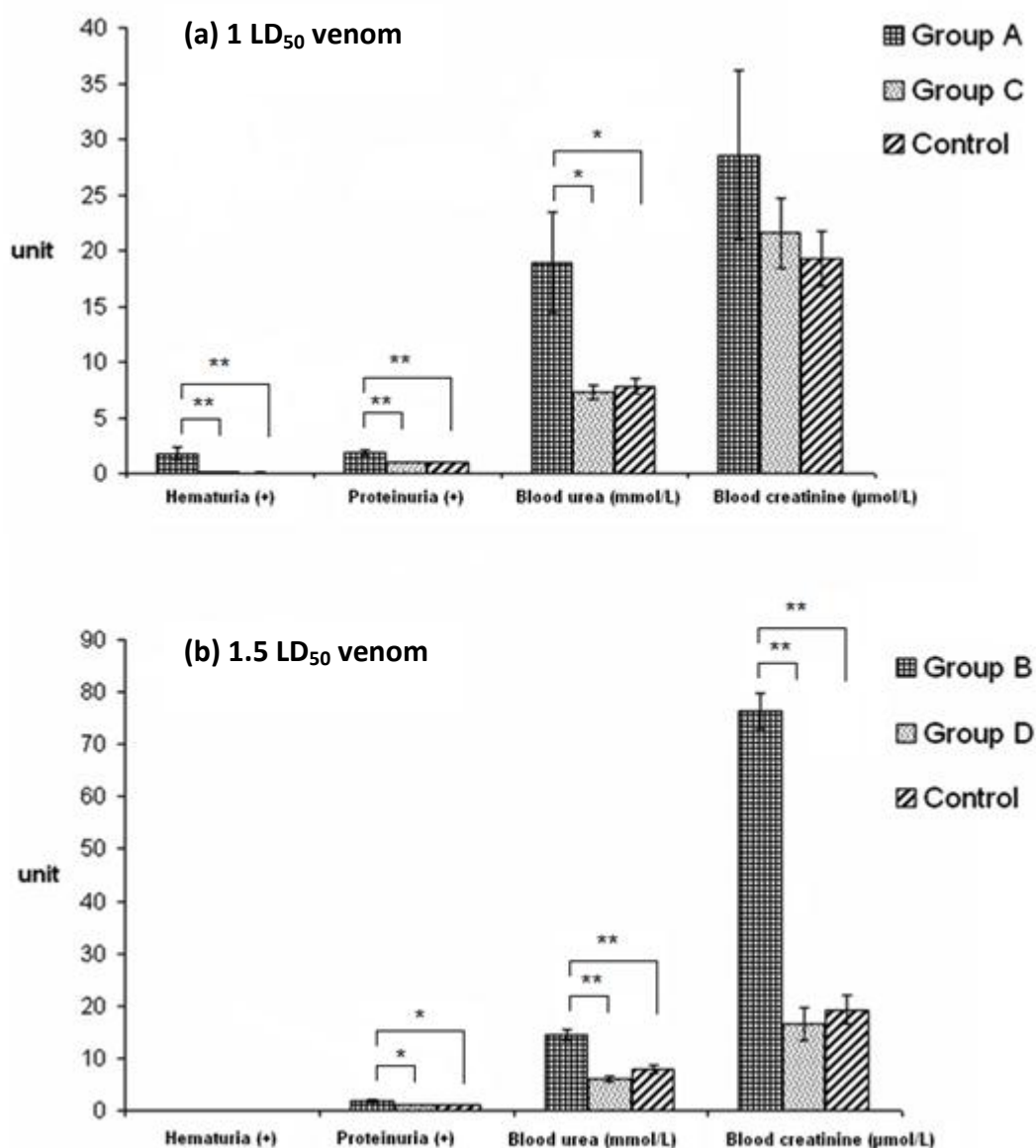


Figure 6.2 Effect of Hemato polyvalent antivenom on the urine and blood biochemical parameters of mice envenomed with *Hypnale hypnale* venom at (a) 1 LD₅₀, and (b) 1.5 LD₅₀. Group A was challenged with 1 *i.p.* LD₅₀ (n = 9, 55% death); (b) Group B was challenged with 1.5 *i.p.* LD₅₀ (n = 4, 100% death). Group C and Group D were the intervened groups which received *i.v.* 200 μL of antivenom (HPA) 5 minutes after envenomation for each mouse. Control was treated with normal saline instead of venom and antivenom. Data were expressed as means ± SEM. Significant differences of mean and between the challenged group and the intervened and/or control groups were indicated as * ($p \leq 0.05$) or ** ($p \leq 0.001$) over the bars, determined by one-way ANOVA and Tukey's post hoc test. No significant difference of means was noted between the intervened groups and the corresponding controls.

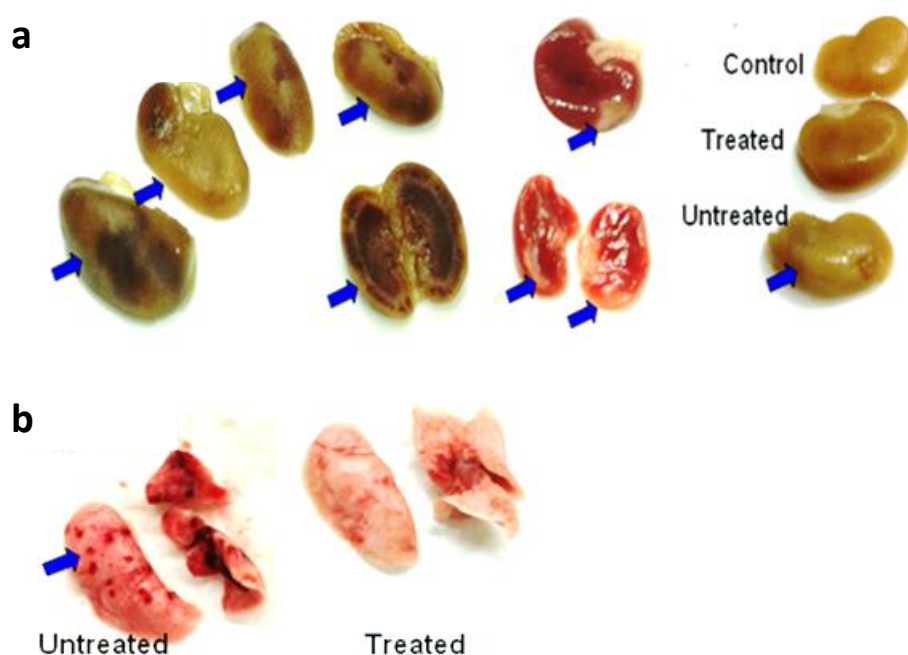


Figure 6.3 Effect of Hemato polyvalent antivenom treatment on the venom-induced gross pathologies in (a) kidneys, and (b) lungs of mice envenomed with *Hypnale hypnale* venom at 1.5 LD₅₀. In (a), arrows indicate patches of pallor on renal cortices from mice challenged with *H. hypnale* venom. Note the absence of pallor in the treated and control specimens. In (b), arrow indicates hemorrhagic spots in the lung of a mouse challenged with the venom. Note the absence of this feature in the treated specimen.

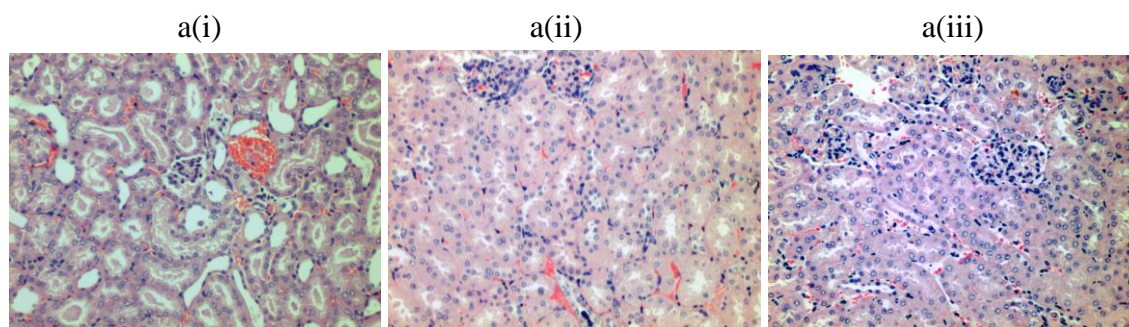
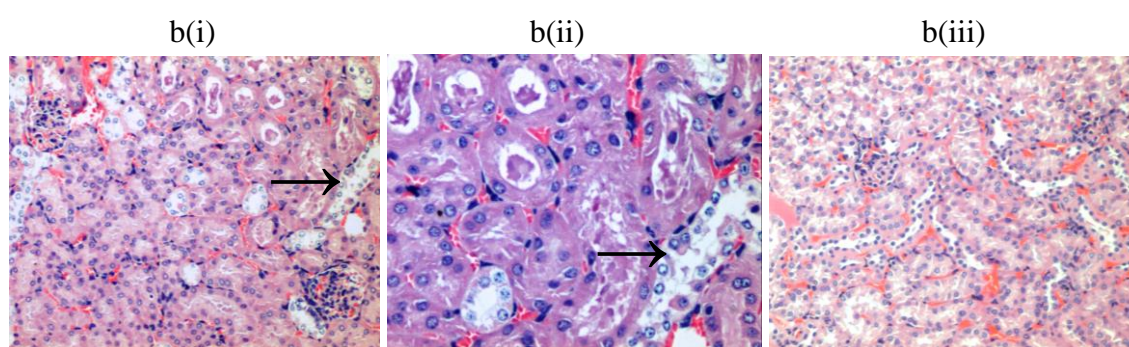
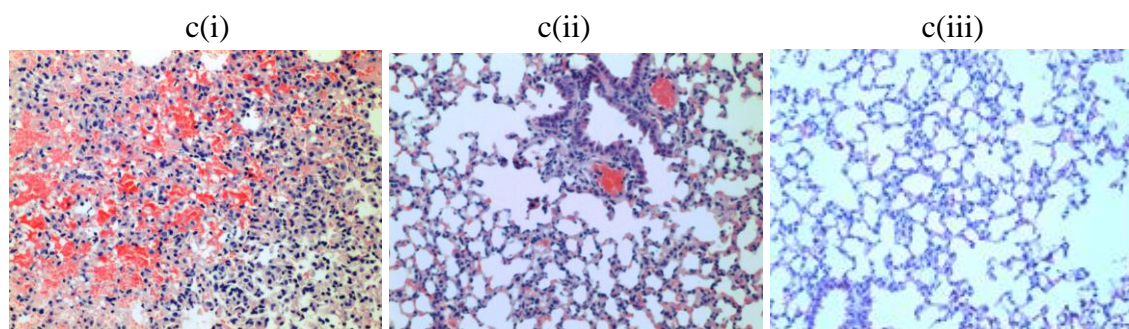
a) Kidney**b) Kidney****c) Lung**

Figure 6.4 Effect of Hemato polyvalent antivenom on the histology of kidneys and lungs of mice envenomed with *Hypnale hypnale* venom. Representative formalin-fixed, paraffin-embedded tissue sections of a(i) kidney from Group A, a(ii) kidney from Group C, a(iii) kidney from control group, all x100; b(i) kidney from group B, x100, b(ii) kidney from group B, x200, b(iii) kidney from Group D, x100; c(i) lung from Group C, c(ii) lung from Group D, c(iii) lung from control group, all x100.

In a(i), mild degree of tubular dilation and vascular congestion were noted. Image b(i) shows that some epithelial cells were denuded from a tubule (arrow) (enlarged in b(ii)). In c(i), pulmonary hemorrhage was observed with red blood cells filling the bronchiolar and alveolar spaces. These abnormalities were markedly prevented with the treatment of Hemato polyvalent antivenom.

Group A and Group B: Challenge groups (mice injected intraperitoneally with *H. hypnale* venom at 1 LD₅₀ and 1.5 LD₅₀, respectively)

Group C and Group D: Treatment groups (mice received *i.v.* 200 µL Hemato polyvalent antivenom post-envenoming of 1 LD₅₀ and 1.5 LD₅₀, respectively).

Control group: mice received normal saline intraperitoneally

6.3.5 Preliminary study on time-dependent effect of *Hypnale hypnale* venom on mouse renal function and histology

Hypnale hypnale venom at 1 LD₅₀ (6 µg/g) injected intraperitoneally killed 8 out of 20 mice in less than 24 hours. Uremia (increased blood urea), hematuria and proteinuria were noted as early as day 2 post-challenge in mice surviving *H. hypnale* experimental envenoming. While the blood urea level seemed to decrease on the following days, the blood creatinine level increased most significantly from day 2 to day 5 ($p < 0.05$), accompanied by worsening hematuria and proteinuria ($p < 0.01$) in the surviving mice. Observation on the animals revealed typical signs of inactivity, slow and shallow breathing, and lack of response to manipulation in the beginning course of envenoming, lasted up to 3-5 days. The signs generally improved from day 5 onwards, accompanied by an improvement in the blood and urine biochemistry of the surviving mice. Histological examination revealed no remarkable changes on the kidneys of the mice that survived the envenomation from day 2 to day 21.

The results from the 12 surviving mice which represented time-dependent effect of the venom at day 5, 10, 15 and 21 post-envenoming are shown in Figure 6.5 (urine and blood biochemistry) and Figure 6.6 (histology of kidneys). The results from mice surviving at day 2 ($n = 4$) and from the control group ($n = 4$), adapted from Section 6.3.2, were included in Figure 6.5 for comparison.

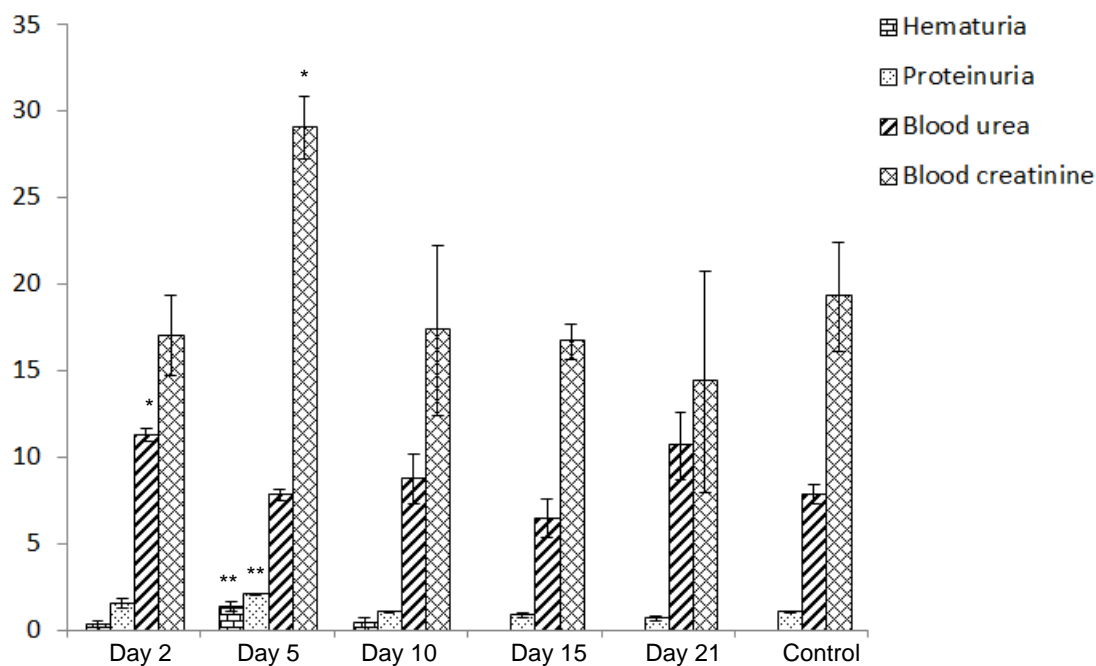


Figure 6.5 Time-dependent effect of *Hypnale hypnale* venom (1 LD₅₀) on urine and blood biochemical parameters of mice that survived the venom challenge. Control was treated with normal saline instead of venom and antivenom. Data were expressed as means \pm SEM. Significant differences of mean between the challenged group and the control group were indicated as * ($p \leq 0.05$) or ** ($p \leq 0.001$) over the bars, determined by Student t-test.

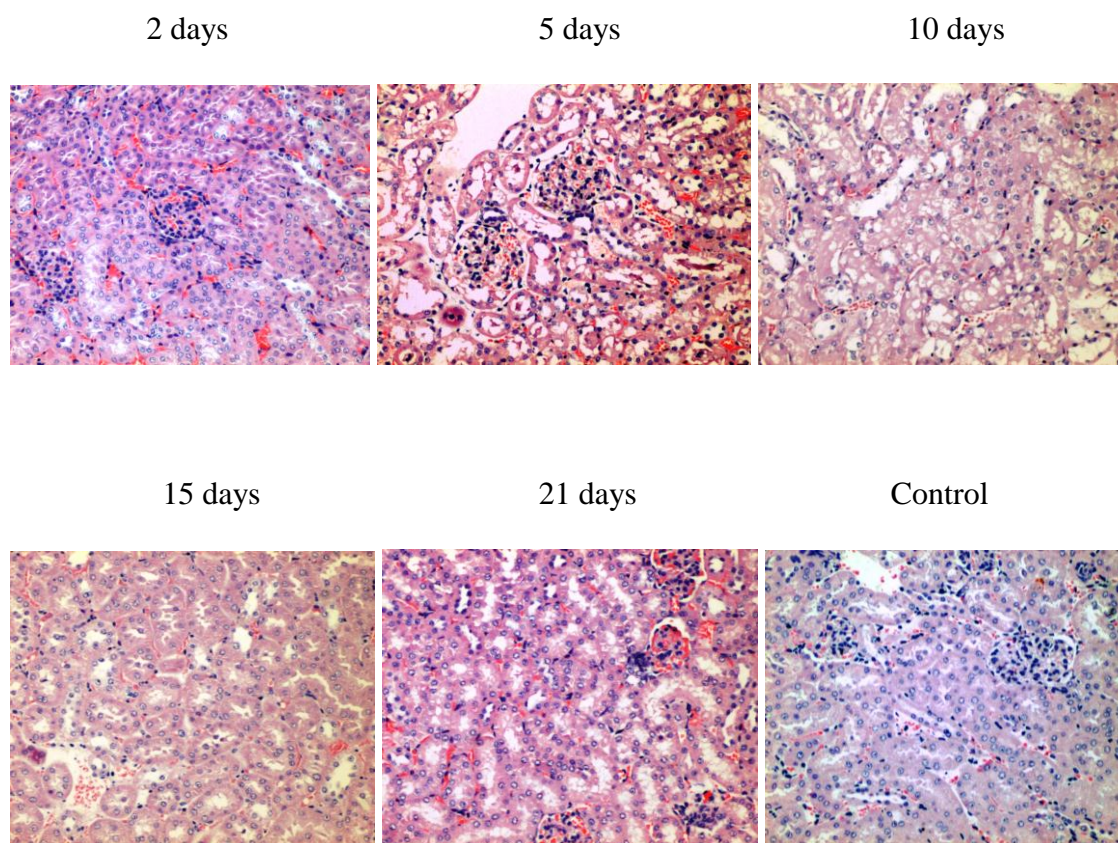


Figure 6.6 Time-dependent effect of *Hypnale hypnale* venom (1 LD₅₀) on mouse kidney histology. Representative formalin-fixed, paraffin-embedded tissue sections of kidneys (x100) from mice survived *H. hypnale* venom injection at 1 LD₅₀. Kidneys were harvested at different time points (days) following envenomation. There were no remarkable changes on the renal histology under light microscopy over the time course in these surviving mice.

6.4 DISCUSSION

6.4.1 Nephrotoxic effect of *Hypnale hypnale* venom

6.4.1.1 A preliminary study in rats with sublethal dose

In this study, *D. siamensis* venom from Myanmar was used in the positive control group (DV group) to produce acute kidney injury (AKI) as the venom was known to be highly nephrotoxic (Ratcliffe *et al.*, 1989; Soe *et al.*, 1993; Willinger *et al.*, 1995; Chaiyabutr and Sitprija, 1999). The blood urea and creatinine, used as biochemical indicators for renal failure (as in acute kidney injury), however, showed no remarkable findings probably due to the fact that the observation period was not sufficiently long. Nevertheless, the significant proteinuria and hematuria are sufficient indication that the *D. siamensis* venom injected did cause acute nephrotoxicity in the animals. The absence of these features in rats injected with similar sublethal dose of *H. hypnale* venom suggested that *H. hypnale* venom may not cause direct nephrotoxicity at the given dose of the venom. This is further demonstrated by light microscopic examination of the histology of kidney and other vital organs (heart, lung, liver, spleen and diaphragm) of rats injected with the sublethal dose of *H. hypnale* venom, where all tissues appeared histologically intact. Thus, a tentative explanation of nephropathy observed in a number of victims (da Silva *et al.*, 1979; Ariaratnam *et al.* 2008) is that the kidney damage is likely the result of complications from the venom hematoxic effects mediated through its procoagulant and fibrinolytic actions, causing renal insufficiency. This suggestion is in line with the view that the clinical nephropathy was likely associated with disseminated intravascular coagulopathy (Sitprija, 2008).

Gunatilake *et al.* (2003) observed epithelial cellular necrosis in the proximal convoluted tubules of rabbit kidney slices that were incubated at 10 mg/ml venom concentration for 3-4 hours, and suggested that hump-nosed pit viper venom had direct nephrotoxic effect on kidney cells. The conclusion is, however, questionable as the necrotic effect was only induced by venom at extremely high concentration (10 mg/mL). In fact, using the same methodology, the report also showed that the local cobra venom caused the most severe degree of direct nephrotoxic effect (even more so than that caused by Russell's viper and hump-nosed pit viper venoms) – a feature not clinically reflected in actual cobra-bite cases. Their findings, nevertheless, indicated that there are substantial components, likely cytolytic enzymes, present in the venom that may be able to cause direct cell death under specific conditions. In view of the venom's prominent proteolytic, necrotic and cytotoxic activities (Maduwage *et al.*, 2011; Chapter 4: Section 4.3.4-4.3.5), a direct tissue-damaging effect on the kidney, or indirect immunological responses induced by the venom at higher doses cannot be completely ruled out at this stage.

6.4.1.2 Extended tests in mice with lethal doses

This study was a continuation of the preliminary nephrotoxicity study in rats. The methodology was modified for the choice of animal (mice instead of rats) and route of venom administration (intraperitoneal instead of intramuscular route) to minimize the amount of venom used in view of the explorative nature of the study. The challenge dose was increased from 1/3 LD₅₀ to 1 LD₅₀ and 1.5 LD₅₀; while the observation period was also lengthened from 24 h to 48 h to allow for the development of a more prominent nephrotoxic feature.

H. hypnale venom at median lethal dose (LD₅₀) (Group A) caused blood biochemical derangement suggestive of acute renal failure, most prominently uremia (and observably increased serum creatinine level). Elevated blood urea level (directly proportionate to blood urea nitrogen) generally indicates a moderate-to-severe degree of renal failure; and this is supported by the development of hematuria and proteinuria that correlated with the deteriorating renal function and signs observed in mice dead by 24 h.

In mice challenged with 1.5 LD₅₀ (Group B), severe azotemia (with blood creatinine increased much higher than urea) observed in all mice indicated that the renal function was further compromised. While significant proteinuria and prolonged clotting time were observed, there was however no hematuria detected, probably because glomerular involvement was not extensive in view of the shorter period of time to death. Pallor noted on the renal cortices was suggestive of renal insufficiency and probable renal ischemia, supported by early histological changes of acute tubular necrosis although the abnormal histology was not extensive and did not involve all animals. This suggests that electronic microscopic examinations may be necessary for a more thorough investigation of subcellular ultrastructural changes in the mouse model. On the other hand, it appears that the nephrotoxic effect of the venom was associated with bleeding disorders shown by prolonged clotting time and lung hemorrhage. The increased urine specific gravity of the mice constituted a supportive finding indicative of pre-renal azotemia, which is likely a complication of reduced renal perfusion secondary to altered hemodynamics.

The result in general suggests that death in mice experimentally envenomed with *H. hypnale* venom was associated with AKI in a background of deranged hemostasis, consistent with several clinical reports (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008).

6.4.2 Neutralization of nephrotoxicity of *Hypnale hypnale* venom with antivenom

Although antivenom remains as the definite treatment for snakebite envenoming (Chippaux and Goyffon, 1998), there is currently no effective antivenom clinically available to treat *H. hypnale* envenoming. However, in laboratory mice, the Hemato polyvalent antivenom (HPA) was proven to be effective in neutralizing the lethal, hemorrhagic and necrotic effects of this venom, in addition to neutralization of procoagulant activity on bovine fibrinogen and human citrated plasma (Chapter 5). HPA thus may be a potential therapeutic solution for the endemic medical urgency caused by *H. hypnale* envenoming, although a controlled clinical trial will be needed eventually to validate its effectiveness in human victims. Nevertheless, acute kidney injury (AKI), observed in approximately 10% of cases (Ariaratnam *et al.*, 2008), is a severe complication known to be the most likely fatal cause in *H. hypnale* envenomation. Clinically, although AKI complicating *H. hypnale* bite were often reversible with timely acute care (demanding dialysis), some surviving patients eventually progressed into chronic kidney disease with ensuing death within a year, simply because of the lack of affordable renal replacement therapy in a developing country that is socioeconomically constrained (Ariaratnam *et al.*, 2008). Therefore, any antivenom therapy proposed for the treatment of *H. hypnale* bite needs to address the issue of venom nephrotoxicity, in addition to its neutralizing efficacy on other major hematoxic effects, and above all, lethality. In this context, Hemato polyvalent antivenom (HPA) was shown to effectively prevent death and AKI in the *in vivo* rodent model, evidenced by the normalization of blood and urine biochemistry as well as kidney and lung anatomy of the envenomed animal. The mechanism of nephrotoxicity neutralization likely involved the HPA cross-neutralizing the procoagulant and

hemorrhagic effects of *H. hypnale* venom (as shown in Chapter 5), hence preempting AKI, a complication associated with consumptive coagulopathy (Sitprija, 2008). The cross-neutralization observed was in fact also supported by immunological cross-reactivity study (Chapter 8), which reflects similarity of antigenic properties between the venoms of *H. hypnale* and *Calloselasma rhodostoma* venoms. However, venom-induced defibrinogenation complicating AKI is not a known feature in *C. rhodostoma* envenoming (Ho *et al.*, 1986a), hence implying subtle differences in the biochemical aspects of the procoagulant enzymes from these two crotalid venoms, possibly at the fibrinolytic mechanism. Essentially, the current data based on *in vivo* animal experiment complements the cross-neutralization study reported previously on the venom's hematoxic and lethal effects, hence supporting the ability of HPA to confer paraspecific protection, and justifying the suggestion for its clinical trial in the future.

6.4.3 Time-dependent nephrotoxic effect of *Hypnale hypnale* venom

In the group of mice challenged with *H. hypnale* venom at 1 LD₅₀ (Group A), death with AKI occurred as early as 11 h post-envenoming, while the nephropathic features (proteinuria, hematuria, uremia) persisted in the surviving mice. Following the time course profiles of the biochemical indicators, it might be concluded that acute kidney injury (AKI) developed as early as few hours post-envenomation, progressed to azotemic state from day 2 to day 5 where hematuria still persisted in the surviving mice. The abnormalities however appeared to be reversible in mice, as the increase in blood creatinine, hematuria and proteinuria were subsequently normalized by day 10 to day 15, consistent with the improvement in the signs of the animals. The histology of the kidneys of the surviving mice was unremarkable throughout the observation period up to 21 days. Usually, histological changes of tubular epithelium are more subtle in the

ischemic type than that in the toxic injury; and altered epithelial structure is often accompanied by a repair as expected in view of the reversible renal dysfunction, thus causing a less conspicuous necrotic feature of the cells when examined under light microscopy.

The findings implies that serum and urine biochemical abnormalities may present in the initial course of envenoming of *H. hypnale* and warrant close monitoring for early detection of nephrotoxicity, in addition to urine output monitoring (for oliguric or anuric types of AKI). Although spontaneous recovery was observed in surviving mice in the current rodent model, clinically appropriate and early supportive intervention (hydration, renal dialysis) are likely the most essential measures at this stage to ensure full recovery before the tissue injury is profound enough to progress into chronic kidney disease. The prognosis, nevertheless, is hoped to improve in the future with the introduction of effective antivenom as the definite treatment that pre-empts the onset of AKI altogether.

CHAPTER 7

Immunological Properties of *Hypnale hypnale* venom: Immunogenicity and Antibody Production towards Potential Therapeutic Application

7.1 INTRODUCTION

Extensive immunological studies on snake venoms over a century clearly showed that snake venoms are complex mixtures of immunogenic components that are able to induce antibody production in animals. Most “anti-venom” immunoglobulins (antibodies) are produced by hyperimmunization of mammals (most commonly, horses and rabbits for therapeutic and diagnostic purposes, respectively), and the products are often polyclonal immunoglobulin G (IgG). The anti-venom antibodies ideally can bind in a specific manner to venom antigens, forming immunocomplexes and thus neutralizing the lethal and other toxic effects of venoms. They serve as the most fundamental biological compounds from which therapeutic antivenoms are derived. The production of antibody *in vivo* and the antibody potency in neutralizing venom toxic effects, however, vary according to the source of venom used, the inherent immunogenic potential of particular toxins and, to a certain degree, the type of host animals employed. When used against heterologous venom, the ‘variation’ in terms of antivenom efficacy is usually even bigger due to the paraspecific nature of a heterologous antivenom, presumably, with suboptimal binding of various toxins originated from a different species. Although certain paraspecific antivenoms may be effective against some heterologous venoms, conceptually, a species-specific antivenom would be able to recognize and bind more toxin antigens than a paraspecific one, as the antivenom is optimally raised against the homologous venom to ensure a wider spectrum of toxins to be neutralized. This chapter discusses the work on the immunogenic response induced by *Hypnale hypnale* venom in rabbits, and the neutralizing potency of the resultant antibody against the lethal and toxic effects of the venom.

7.2 METHODS

7.2.1 Immunization

Rabbits ($n = 3$) of 2 kg each were initially immunized intramuscularly over the back muscles with *H. hypnale* venom (50 μg in 0.5 mL PBS, pH 7.4) emulsified in 0.5 mL complete Freund's adjuvant, followed by three subsequent intramuscular doses of 100 μg venom in 0.5 mL PBS emulsified in 0.5 mL incomplete Freund's adjuvant at fortnightly intervals.

Blood (~2 mL) was collected via the rabbits' marginal ear veins, just before each immunization, including the first dose. The blood sample was left to clot in plain tubes at room temperature, and subsequently centrifuged at 3,000 g to separate the serum. From the serum (antiserum), the antibody titer levels (at two weeks following each injection) were monitored by indirect ELISA (Section 7.2.2).

7.2.2 Indirect ELISA for determination of antibody production

Microtiter wells were each coated with 100 μL venom of *H. hypnale* (100 ng/mL in carbonate-bicarbonate buffer, pH 9.6) overnight at 4 °C. After washing with 100 μL PBS-Tween 20 for four times, each well was incubated with 100 μL serum samples diluted 1:400 in PBS-Tween 20, pH 7.4, for 1 h at room temperature. Following another cycle of washing, goat anti-rabbit IgG-HRP in PBS-Tween 20 (1:3000 dilution made up to 100 μL) was added and left for 1 h at room temperature. The HRP activity was commenced after washing by incubation with substrate o-phenylenediamine (OPD, 0.4 mg/mL and 0.006% H_2O_2 in 0.1 M citrate-phosphate, pH 5) for 30 min at room

temperature in the dark. The reaction was terminated by addition of 50 μL of 12.5% H_2SO_4 and the absorbance was read at 492 nm with a microplate reader (SUNRISE-TECAN Type Touch Screen F039300). All experiments were performed in triplicates.

7.2.3 Purification of immunoglobulins G (IgG)

Antisera showing highest antibody titer (as monitored by ELISA) were collected from rabbits by cardiac puncture under anesthesia (intramuscularly injected cocktail of xylazine and ketamine, at 5 mg/kg and 50 mg/kg body weight of rabbit, respectively).

The purification of anti-*H. hypnale* (anti-Hh) IgG was then carried out with protein precipitation technique using caprylic acid. The procedure was carried out according to Rojas et al. (1994). Caprylic acid was mixed with 10 mL anti-Hh serum to a final concentration of 5% at pH 6. Following vigorous stirring for 1 h at room temperature, the mixture was centrifuged at 10,000 g for 10 min, and the supernatant was collected and diafiltered with distilled water several times using VivaspinTM concentrator (with molecular weight cut-off point of 30,000 kDa). The IgG in distilled water was then lyophilized, and the product was kept at -20 °C for later use in venom neutralization studies.

7.2.4 Determination of lethality, hemorrhagic, procoagulant and necrotic activities

The value of intravenous median lethal dose (LD_{50}) of *H. hypnale* venom in mice was based on that described in Chapter 4 (Section 4.3.2). Minimum hemorrhagic dose (MHD, the dose that caused a hemorrhagic lesion diameter of 10 mm), minimum coagulant dose (MCD, the minimal dose that clots a standard solution of bovine

fibrinogen in 60 s), and the minimum necrotic dose (MND, the minimal dose that induces a necrotic lesion with a diameter of 5 mm) were based on previous determinations from Chapter 5 (Section 5.1.2-5.1.4).

7.2.5 *In vitro* neutralization of the venom lethality, hemorrhagic, procoagulant and necrotic activities by anti-Hh serum and IgG

These were carried out as modified from Ramos-Cerrillo et al. (2008) and made comparable to the work described in Chapter 5 (see 5.3.2).

Challenge doses for lethality, procoagulant, hemorrhagic and necrotic effects were respectively 2.5 LD₅₀ (for antiserum) or 5 LD₅₀ (for IgG), 2 MCD, 2 MHD and 2.5 MND. In brief, the venom was mixed thoroughly with various dilutions of antiserum or IgG solution (made up to 50 µL for intradermal injections; and 200 µL for intravenous injections). The IgG dilution was carried out according to MPVA/HPA instruction manual where 1 vial of ~1g antivenom is to be reconstituted in 10 mL solution. The mixtures of venom and antiserum or IgG were then incubated at 37 °C for 30 min with gentle shaking, following which tests for the corresponding toxic activities were carried out. The effective doses (ED or ED₅₀) were calculated as described in Chapter 5 (Section 5.2.2.1-5.2.2.4). In addition, potency of the antivenom, P, defined as the amount of venom (expressed in number of median lethal doses) that is completely neutralized per unit volume of antivenom (specifically for lethality test), was calculated according to the European Directorate for the Quality of Medicines (1997) and Araujo et al. (2008) as follows:

$$P = (n-1) LD_{50} / ED_{50}$$

where LD_{50} is the median lethal dose and n is the number of LD_{50} used in the challenge assay. The expression $(n-1)$ is used instead of the total number of n , because at the endpoint of the neutralization assay, 1 LD_{50} remains unneutralized and causes the death of 50% of mice.

7.3 RESULTS

7.3.1 Immunization and antiserum production

The antigenicity of *H. hypnale* venom was expressed with a rapid rise of titer level in 2 weeks following the primary immunization (Fig. 7.1). The antibody titer level subsequently rose steadily towards the third immunization, following which it plateaued on subsequent booster. The final level upon completion of the immunization was noted to be slightly lower than the previous one but the difference was not statistically significant.

7.3.2 Neutralization of the lethal and toxin effects of *Hypnale hypnale* venom with anti-*Hypnale hypnale* (anti-Hh) serum and IgG

Preliminary tests with 5 LD₅₀ of *H. hypnale* venom incubated with 200 µl antiserum unfortunately killed most mice. When tested against 2.5 LD₅₀ in mice, the antiserum nevertheless appeared effective in neutralizing the lethality of the venom with an ED₅₀ of 97.6 µL (95% C: 46.6-187.2 µL). The IgG isolated using caprylic acid precipitation method showed a more superior neutralizing efficacy than that of the antiserum, with an ED₅₀ of 83.1 µL (95% CI: 40.9-176.8 µL) when tested against the lethal effect of 5 LD₅₀. Its neutralizing efficacy was comparable to that of the monovalent Malayan pit viper antivenom (MPAV), but less potent than that of Hemato polyvalent antivenom (HPA). Table 7.1 summarizes the data on neutralizing efficacy against lethality for the different types of immunological derivatives tested thus far for comparison.

The anti-Hh sera were effective to neutralize the procoagulant, hemorrhagic and necrotic activities of the venom *in vitro*. The efficacies of neutralization were however significantly increased with use of the anti-Hh IgG ($p < 0.05$). Nevertheless, the effective doses of anti-Hh IgG against the three common toxic effects of viperid venoms were significantly higher (lower potencies) compared to those of HPA ($p < 0.05$), a finding in line with the result from lethality neutralization test. The IgG was also slightly less potent than MPVA in neutralizing the procoagulant and hemorrhagic effects, but not the necrotic effect where both were equally effective.

Table 7.2 shows the *in vitro* neutralizing effective doses of the different types of immunological derivatives on procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom.

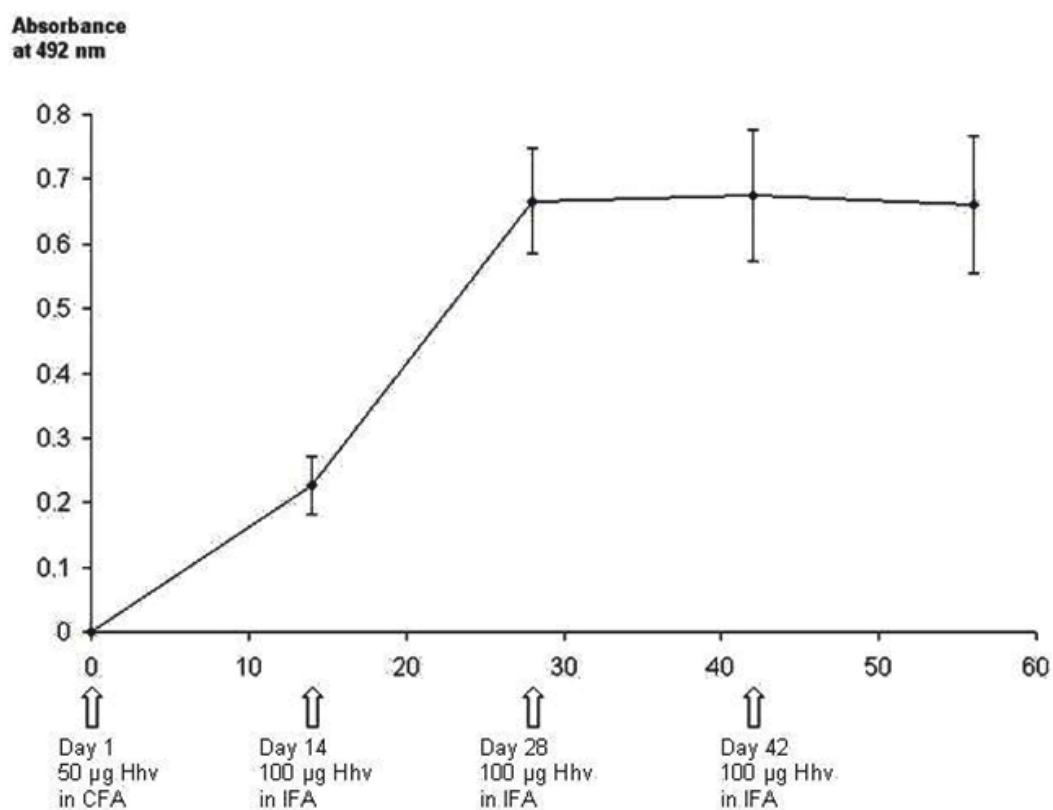


Figure 7.1 Immune response elicited in three rabbits monitored by indirect ELISA following a fortnightly immunization schedule. Arrows show the days of immunization with contents of injection: Hhv – *H. hypnale* venom; CFA: Complete Freund's adjuvant; IFA: Incomplete Freund's adjuvant. Values were mean absorbance \pm SEM.

Table 7.1 Neutralization of the lethal effect of *Hypnale hypnale* venom by the monovalent Malayan pit viper antivenom, Hemato polyvalent antivenom, anti-*Hypnale hypnale* sera and anti-*Hypnale hypnale* IgG.

Antivenom	ED ₅₀ (95% CI.) (μL antivenom/challenge dose)	Potency, P (LD ₅₀ per mL)
Monovalent Malayan Pit Viper antivenom (MPVA)	70.7 μL* (33.7-148.4)	57
Hemato Polyvalent antivenom (HPA)	41.5 μL* (20.4-88.4)	96
Anti- <i>Hypnale hypnale</i> sera	97.6 μL** (46.6-187.2)	15
Anti- <i>Hypnale hypnale</i> IgG	83.1 μL* (40.9-176.8)	48

* Venom challenge dose = 5 LD₅₀, ** Venom challenge dose = 2.5 LD₅₀. The i.v. LD₅₀ of *H. hypnale* venom (0.9 μg/g), ED₅₀ of MPVA and HPA were based on previous findings in Chapter 5.

Table 7.2 Neutralization of the procoagulant, hemorrhagic and necrotic activities of *Hypnale hypnale* venom by the monovalent Malayan pit viper antivenom (MPVA), Hemato polyvalent antivenom (HPA), anti-*Hypnale hypnale* sera (anti-Hh sera) and anti-*Hypnale hypnale* IgG (anti-Hh IgG).

Toxic activity	ED or ED ₅₀ (MPVA) μL/mg	ED or ED ₅₀ (HPA) μL/mg	ED or ED ₅₀ (anti-Hh sera) μL/mg	ED or ED ₅₀ (anti-Hh IgG) μL/mg
Procoagulant ^a	432.1 ± 10.9	114.2 ± 2.7	644.1 ± 8.1	556.36 ± 12
Hemorrhagic ^b	472.3 ± 13.9	67.4 ± 5.1	781.00 ± 11.4	618.50 ± 12
Necrotic ^c	612.6 ± 17.5	53.8 ± 0.5	646.0 ± 20.5	615.59 ± 11

^aMinimal procoagulant dose: 56.2 ± 1.3 μg/mL

^bMedian hemorrhagic dose: 10.5 ± 0.2 μg

^cMedian necrotic dose: 39.3 ± 1.6 μg

The minimal doses of procoagulant, hemorrhagic and necrotic activities, as well as the effective doses of MPVA and HPA were based on the previous findings stated in Chapter 5. Values were expressed as mean ± SEM.

7.4 DISCUSSION

7.4.1 Immunogenicity and antibody production for *H. hypnale* venom

In the current study, immunization of animals with *H. hypnale* venom was carried out for the first time, and the satisfactory antibody titer rising indicated that the venom was sufficiently antigenic to generate a good immune response in the animals. Some important factors associated with immunogenicity of antigens have been considered for the immunization: rabbits (host animal) certainly have a very distant phylogenetic relationship with the snake (source of antigen), while the venom is generally made up of proteins and peptides with high complexity. Viperid venoms are known to be qualitatively and quantitatively different from many elapid venoms, with the predominant presence of high molecular weight components especially hydrolytic enzymes, e.g. serine proteases and metalloproteases (Serrano et al., 2005; Mackessy, 2010a). *H. hypnale* venom has been shown to exhibit various enzymatic activities, notably the thrombin-like enzyme, proteolytic enzyme, hyaluronidase, L-amino acid oxidase and phospholipase A₂ (Chapter 4). These toxic protein components are medium to large molecules (molecular weights of 14-28 kDa and above) which generally elicit greater antibody production, as immunogenicity is known to increase with the size of the antigen (Harlow and Lane, 1988). The use of adjuvant in the immunization scheme also served to augment the antigen complexity, hence enhancing the immune response and antibody production (Herbert, 1928), demonstrated by the rapid rise in antibody titers in the first three immunizations. Plateauing of the antibody titer level thereafter implied that the immune response was mature and the antibodies produced were essentially immunoglobulins G from the secondary response. The results suggest that in production of antivenom against *H. hypnale* venom, a short duration (3 immunizations

in total) may be adequate based on the described protocol, hence reducing the amount of venom required for antivenom production. This is an important consideration as supply of *H. hypnale* venom is always limited due to the tiny body size of the snake (Joseph et al, 2007; also see 4.4.1 of Chapter 4,).

7.4.2 Neutralizing efficacy of anti-Hh serum and IgG as species-specific antivenom

Currently, the Hemato polyvalent antivenom (HPA) has been suggested for therapeutic use in endemic Sri Lanka where antidotes are urgently needed to abate the envenoming crisis (Christeine A. Gnanathan, personal communication, 26 June 2011) in view of its capability of cross-neutralizing *H. hypnale* venom (Chapter 5). However, the antivenom is yet to be assessed for its clinical efficacy in human patients. It is presumed that in paraspecific protection, though effective, not all toxins of the *H. hypnale* venom will be neutralized at optimum by a heterologous antivenom. Conceptually, the production of specific antivenom using the homologous *H. hypnale* venom is therefore essential and warranted; and long-term efforts should be undertaken for this industry. To apply such therapeutic principle, two pertinent issues need to be addressed: (a) the antigenicity of the venom for antibody production, and the resultant antibody potency in neutralizing the venom; (b) the identification of biting species for an accurate immunotherapy. It is known that *H. hypnale* has been misidentified especially as *Echis carinatus* (saw-scaled viper), or occasionally as *Daboia russelii* (Russell's viper) – two common biting snakes in those areas, hence complicating the clinical management (Joseph et al., 2007, Ariaratnam et al., 2009). The latter issue is addressed in Chapter 8.

In the current study, the crude anti-Hh serum failed to neutralize the lethal effect of high dose *H. hypnale* venom (5 LD₅₀ as recommended by the European Pharmacopoeia

(1997) and adopted by most authors), but appeared moderately effective when the challenge dose was reduced to 2.5 LD₅₀. Such discrepancy could be explained by the fact that venom-antivenom dose-response relationship does not necessarily follow linearity at higher doses of venom, as was also observed by Bogarín et al. (2010). In another word, venom-antivenom neutralization study does not necessarily follow the ‘law of multiple proportions’, since different venom toxins may exert a ‘relevant’ (medically significant) role at different challenge doses (Christensen, 1966), hence the complexity of *in vivo* effects induced at different doses. The expression of the efficacy of antivenom should be therefore standardized for consistency: by avoiding ambiguity from using ED₅₀ alone when comparing neutralization efficacy of various products against different ‘n’ (numbers) of venom LD₅₀. The parameter ‘potency’, P, that represents the property of a product in conferring 100% protection against lethality, was adopted for this reason (Morais et al., 2010).

The neutralizing efficacy of anti-venom product certainly relies on the density of the paratopes (or fragment of antigen binding) of immunoglobulins to which toxin antigens bind. Caprylic acid precipitation, a common and effective way of yielding IgG from crude serum in large-scale antivenom production (Rojas et al., 1994; Raweerith and Ratanabanangkoon, 2003; Khomvilai, 2008) by precipitating the non-IgG proteins from the antiserum could have improved the purity and amount of IgG in the current study, hence explaining the higher potency than the antiserum one. Caprylic acid precipitation method was used in this study as to mimic the production of the monovalent Malayan pit viper antivenom (MPVA) and Hemato polyvalent antivenom (HPA) by the Thai Red Cross Society from hyperimmunized equine sera. The efficacy of paraspecific neutralization by the mentioned antivenoms has been proven and discussed (Chapter 5). However when comparing these two commercial paraspecific antivenoms to the

homologous IgG, the IgG, although being species-specific, was generally less potent especially than the HPA. The observation indicates that HPA at a given volume contained higher fractions of IgG paratopes which could recognize and bind to the *H. hypnale* venom toxin antigens. This is either due to the additional treatment during HPA manufacturing, i.e. pepsin digestion of the antibodies from hyperimmunized horses yielding concentrated (90%) F(ab')₂ fragments (Khomvilai, 2008), or due to the shared antigenic properties of components from *D. siamensis* and/or *C. albolabris* venoms used together with *C. rhodostoma* venom as immunogens in the production of HPA. This is supported by the observation that anti-Hh IgG cross-reacted substantially with *C. albolabris* venom in both indirect and double-sandwich ELISA (see Chapter 8). The efficacy of anti-Hh IgG in neutralizing the procoagulant and hemorrhagic activities was also slightly lower than that of the monovalent MPVA, which also had undergone pepsin digestion of its antibody. At this stage we were unable to test the various parameters to optimize caprylic acid precipitation and pepsin digestion as the amount of antisera/IgG collected were limited due to the use of small animals (rabbits) for antibody production. However, the results imply that the anti-Hh sera neutralizing capacity for venom toxicity could be improved by caprylic acid precipitation to yield the IgG. Pepsin digestion, initially used to remove allergenic Fc of heterologous antibody (Pope, 1933), should also be considered in future anti-Hh antivenom production for enhancing its therapeutic potency.

The potential application of anti-Hh IgG can also be further explored for the venoms of *Hypnale nepa* and *Hypnale walli*, although envenoming by these two species is far less frequent than that by *H. hypnale*, which is responsible for most reported severe clinical problems (Christeine A. Gnanathan, personal communication, 26 June 2011). While the Hemato polyvalent antivenom (HPA) has been proposed for use in *H. hypnale*

envenoming in view of the medical urgency, the production and refinement of an *H. hypnale*-specific antivenom is nonetheless the therapeutic of choice in the long term. In view of the suboptimal socioeconomic and healthcare system in developing countries, instead of producing a new monovalent antivenom, it is suggested that *H. hypnale* venom to be incorporated into the immunizing regimen for existing polyvalent antivenoms used in the country – for therapeutic coverage of a more complete list of medically important venomous snakes in the region. This however requires further efforts in fine-tuning the optimization for antivenom efficacy especially on a large-scale production, hence representing a research area beyond academic exercise that awaits global partnership with supports in order to overcome various challenges ahead (Gutiérrez et al., 2006).

CHAPTER 8

ELISA Cross-reactivity Study and Application in Diagnostics

8.1 INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) is an analytical biochemical tool that uses one subtype of heterogeneous, solid-phase enzyme immunoassay (EIA) to detect the presence of a substance (usually an antigen) and where possible, to quantitate it. Its use in the study of snake venoms has been made popular since the 1980's for detection and quantitation of snake venoms from biological samples of snakebite victims. ELISA has played an important role clinically in confirmatory diagnosis of the biting snake, and for studying venom concentration-time profile during clinical trials. This would be relevant to the current study of *Hypnale hypnale* venom, as misidentification of the snake is not uncommon in the affected region. A proper diagnosis as well as venom quantitation would be essentially necessary in anticipation of future clinical studies. The assay, known for its high sensitivity, is however bound for limitation on specificity. In this chapter, works on purification of antibody and development of an optimized indirect as well as double-sandwich ELISA for *H. hypnale* venom were discussed. The assays were also applied in cross-reactivity study against various venoms, and for studying the immunological titer of *H. hypnale* venom with the monovalent Malayan pit viper antivenom and Hemato polyvalent antivenom. The double-sandwich ELISA was also modified for potential diagnostic as well as venom quantitation use in the future.

8.2 METHODS

8.2.1 Raising antisera of venoms in rabbits

The production and collection of anti-*Hypnale hypnale* sera from rabbits were described in Chapter 7 (Section 7.2.1). Using the same immunization protocols, *Daboia russelii* and *Echis carinatus sinhaleyus* venoms, both of Sri Lankan origin, were used to raise antibodies from rabbits (n = 2 and n = 1 respectively) for double-sandwich ELISA. The antibody titer levels were monitored using indirect ELISA, every two weeks following each injection.

8.2.2 Purification of Immunoglobulins G (IgG)

Antisera showing highest antibody titer (as monitored by ELISA) were collected from rabbits by cardiac puncture under anesthesia (*i.m.* injection of xylazine (1 mg/kg) and ketamine (10 mg/kg). The purification of the IgG of the three venoms (*Hypnale hypnale*, *Daboia russelii* and *Echis carinatus sinhaleyus*) was then performed separately by affinity chromatography using Protein A column.

The antisera were first desalted by Sephadex G-25 gel filtration column to yield the serum protein fractions which were subsequently pooled and freeze-dried. In this desalting method the Sephadex G-25 gel filtration column was packed to a 100 mL volume in a glass column with length 50 cm and inner diameter 2.5 cm connected to Micro Tube Pump MP-3 (Tokyo Rikakidai Co). For every run, 10-15 mL of antiserum was loaded onto the gel column and the serum proteins were eluted using 0.05% acetic

acid at a flow rate of 5 mL/min. The protein fractions were determined at 280 nm absorbance before pooling for lyophilization.

The IgG purification was carried out as described by Hudson and Hay (1980) with slight modifications. One hundred milligrams freeze-dried serum proteins was dissolved in 1 mL 20 mM sodium phosphate (pH 8.0), and applied to the HiTrapTM Protein A-Sepharose gel affinity column (5 mL gel volume) connected to Micro Tube Pump MP-3 (Tokyo Rikakidai Co). Washing of unbound components began with 20 mM sodium phosphate, followed by elution with 0.1 M citric acid pH 3.0 in 1 M Tris-HCl buffer, pH 9.0), at a flow rate of 5 mL/ min. The eluted fractions were monitored at 280 nm absorbance, and the peaks were screened for antibody activity by indirect ELISA (method as outlined in Section 8.4); while the protein purity was monitored by 15% SDS-PAGE under reducing (with 2-mercaptoethanol) and non-reducing conditions. The determined IgG fractions were then pooled, desalted with VivaspinTM concentrator and lyophilized. The freeze-dried IgG was kept at -20 °C for use in double-sandwich ELISA and for horseradish peroxidase conjugation.

8.2.3 Conjugation of IgG with horseradish peroxidase

The assay was performed according to the method of Tijssen (1985). Four milligrams horseradish peroxidase (HRP) was dissolved in 1.0 mL of distilled water and 0.2 mL of freshly prepared 0.1 M sodium periodate was added to it and stirred. The resulting HRP-aldehyde was diafiltered with 1 mM sodium acetate buffer (pH 4.4) at 4 °C for several changes of buffer to reduce auto-conjugation. The pH was then raised to approximately 9.0-9.5 by adding 20 µL of 0.2 M sodium carbonate buffer (pH 9.5). Eight miligrams IgG, dissolved in 1 mL of 0.01 M sodium carbonate buffer (pH 9.5),

was added immediately. The mixture was stirred for 2 h at room temperature, followed by the addition of 100 μ L freshly made sodium borohydride solution (4 mg/mL in distilled water) to reduce the Schiff's base. After 2 h incubation at 4 °C, the IgG-HRP conjugate was diafiltered for buffer exchange with PBS (pH 7.2) at 4 °C, aliquoted and stored at -20 °C.

8.2.4 Indirect ELISA for determination of antibodies

Microtiter wells were each coated with 100 μ L venom of *H. hypnale*, *Daboia russelii* or *Echis carinatus sinhaleyus* respectively (100 ng/mL in carbonate-bicarbonate buffer, pH 9.6) overnight at 4 °C. After washing four times with 100 μ L PBS-Tween 20, each well was incubated with 100 μ L serum samples diluted 1:400 in PBS-Tween 20, pH 7.4, for 1 h at room temperature. Following another cycle of washing, goat anti-rabbit IgG-HRP in PBS-Tween 20 (1:3000 dilution made up to 100 μ L) was added and left for 1 h at room temperature. The HRP activity was commenced after washing by incubation with substrate o-phenylenediamine (OPD, 0.4 mg/mL and 0.006% H_2O_2 in 0.1 M citrate-phosphate, pH 5) for 30 min at room temperature in dark. The reaction was terminated by the addition of 50 μ L of 12.5% H_2SO_4 and the absorbance was read at 492 nm with a microplate reader (SUNRISE-TECAN Type Touch Screen F039300). All experiments were performed in triplicates.

8.2.5 Double-sandwich ELISA for detection of venom antigen

Microtiter wells were each coated with 100 μ L affinity-purified rabbit anti-venom IgG (4 μ g/mL for anti-*H. hypnale*; 5 μ g/mL for anti-*Daboia russelii* and anti-*Echis carinatus sinhaleyus*, respectively, as determined by checkerboard titration) dissolved in

carbonate-bicarbonate buffer, pH 9.6, by incubating overnight at 4 °C. After washing four times with 100 µL PBS-Tween 20, each well was incubated for 2 h at room temperature with 100 µL venom solution (100 ng/mL) dissolved in PBS-Tween 20, or in human sera diluted with PBS-Tween 20 (1:10 dilution made up to 100 µL). Following another cycle of washing, rabbit anti-venom IgG-HRP conjugate, freshly diluted to 100 µL (1:200 for anti-*H. hypnale*; 1:600 for anti-*E. carinatus sinhaleus*; 1:800 for anti-*D. russelii*, determined by checkerboard titration) was dispensed into each well and left for another 2 h at room temperature. The HRP activity was initiated after washing by incubation with 100 µL substrate OPD (0.4 mg/mL and 0.006% H₂O₂ in 0.1 M citrate-phosphate, pH 5) for 30 min at room temperature in the dark. The reaction was terminated by the addition of 50 µL of 12.5% H₂SO₄, and the absorbance was read at 492 nm with a microplate reader (SUNRISE-TECAN Type Touch Screen F039300). All experiments were performed in triplicates.

8.2.6 ELISA cross-reactivity of anti-*Hypnale hypnale* (anti-Hh) serum and anti-Hh IgG with various venoms

Venoms of various species (100 µL of 100 ng/mL solution) were coated on microtiter walls by overnight incubation. Anti-Hh serum (1:400) from the final (4th) immunization and purified IgG (1:500; stock concentration: 1 mg/mL), diluted in PBS-Tween 20 (for both, made up to 100 µL), were each incubated with the pre-coated venoms then for 1 h and the cross-reactivities were determined using indirect ELISA as described in Section 8.2.4.

For double sandwich ELISA, anti-Hh IgG (100 µL of 4 µg/mL solution) was coated on microtiter wells by overnight incubation. A hundred microliters venoms of various

species (100 ng/mL solutions) were used as samples to incubate with the precoated IgG for 2 h, and the cross-reactivities were then determined based on the protocol as described in Section 8.2.5. ELISA cross-reactivities were expressed in mean percentage \pm SEM of the absorbance with reference to reaction between anti-Hh serum or IgG with *H. hypnale* venom.

8.2.7 ELISA cross-reactivity of *Hypnale hypnale* venom with *Calloselasma rhodostoma* monovalent and polyvalent antivenoms (MPVA and HPA)

For indirect ELISA, 100 μ L *H. hypnale* venom (100 ng/mL) was used to coat the microtiter wells overnight. Washing was then done with PBS-Tween 20, and the venom was incubated with 100 μ L antivenoms (MPVA or HPA, with 1:5000 dilution in PBS-Tween 20 made up to 100 μ L) for 1 h. Following another cycle of washing, goat anti-equine IgG-HRP conjugate (1:8000 dilution in PBS-Tween 20 made up to 100 μ L) was used as secondary antibody (1 h incubation) to which the HRP activity on OPD was subsequently measured. A hundred microliters *C. rhodostoma* venom (100 ng/mL) coated on microtiter well was used as the positive control, where homologous venom neutralization has been established in murine model (Chapter 5); while 100 μ L *N. naja* venom (100 ng/mL) coated on microtiter well served as the negative control, where no *in vitro* cross-neutralization has been observed with either MPVA or HPA.

The study was repeated with double-sandwich ELISA, where both coating and primary antibodies were replaced by antivenoms (MPVA or HPA) in 100 μ L coating buffer or PBS-Tween 20 (1:5000 dilution made up to 100 μ L) accordingly. Goat anti-equine IgG-HRP conjugates (1:8000 dilution in PBS-Tween 20 made up to 100 μ L) served as secondary antibodies to which HRP activity on OPD was measured then. The samples

consisted of 100 μ L venoms (100ng/mL) of *H. hypnale*, *C. rhodostoma* (positive control) and *N. naja* (negative control). The protocol was essentially following that described in Section 8.2.5. The cross-reactivities were expressed in mean percentage \pm SEM of the absorbance with reference to the controls.

8.2.8 Detection and quantitation of venoms in human sera

H. hypnale, *E. carinatus sinhaleyus* and *D. russelii* venoms were respectively dissolved in a 1:10 mixture of human serum and PBS-Tween 20, subsequently diluted with the mixture to a series of known concentrations (0-100 ng/mL). These then served as the standard samples for the double-sandwich ELISA as described in Section 8.2.5, under each optimal working condition as predetermined. The absorbance values were then plotted against the standard venom concentrations. Limit of detection (LoD) and limit of quantitation (LoQ) for venom detected in human sera were calculated with the LoD defined as three times (3x) the standard deviation of the blank, and the LoQ defined as ten times (10x) the standard deviation of the blank, with the blank being human serum (1:10 dilution) without venom antigens.

8.3 Results

8.3.1 Production of antisera against the venoms of *Hypnale hypnale*, *Daboia russelii* and *Echis carinatus sinhaleyus*

The production of antisera for *D. russelii* and *E. carinatus sinhaleyus* venoms generally followed the pattern similar to that for *H. hypnale* venom (Figure 7.1), where the antibody titers increased steadily towards the third immunization, following which a plateau was observed (Figure 8.1a and b).

8.3.2 Purification of immunoglobulins G

By a successive utilization of gel filtration and affinity chromatography, polyclonal immunoglobulins G (IgG) were purified from the crude antisera raised against different venoms (*H. hypnale*, *D. russelii*, *E. carinatus sinhaleyus*). Figure 8.2a shows the isolation of serum proteins (commonly known as desalting of antiserum) which were subsequently purified further to immunoglobulins G (Figure 8.2b). The fractions containing IgG (theoretically the eluted fraction from Protein A column) were screened positive for antibody activity by indirect ELISA (with net absorbance > 0.3). The IgG purity monitored by 15% SDS-PAGE under reducing (with 2-mercaptoethanol) and non-reducing conditions was shown as inset in Figure 8.2b. The non-reduced sample presented predominantly as IgG with high molecular mass of ~130-160 kDa. These high molecular weight proteins were however reduced to two main products suggestive of IgG derivatives: the heavy chain with ~50 kDa and the light chain with ~25 kDa. There were no remarkable protein aggregates and albumin detected.

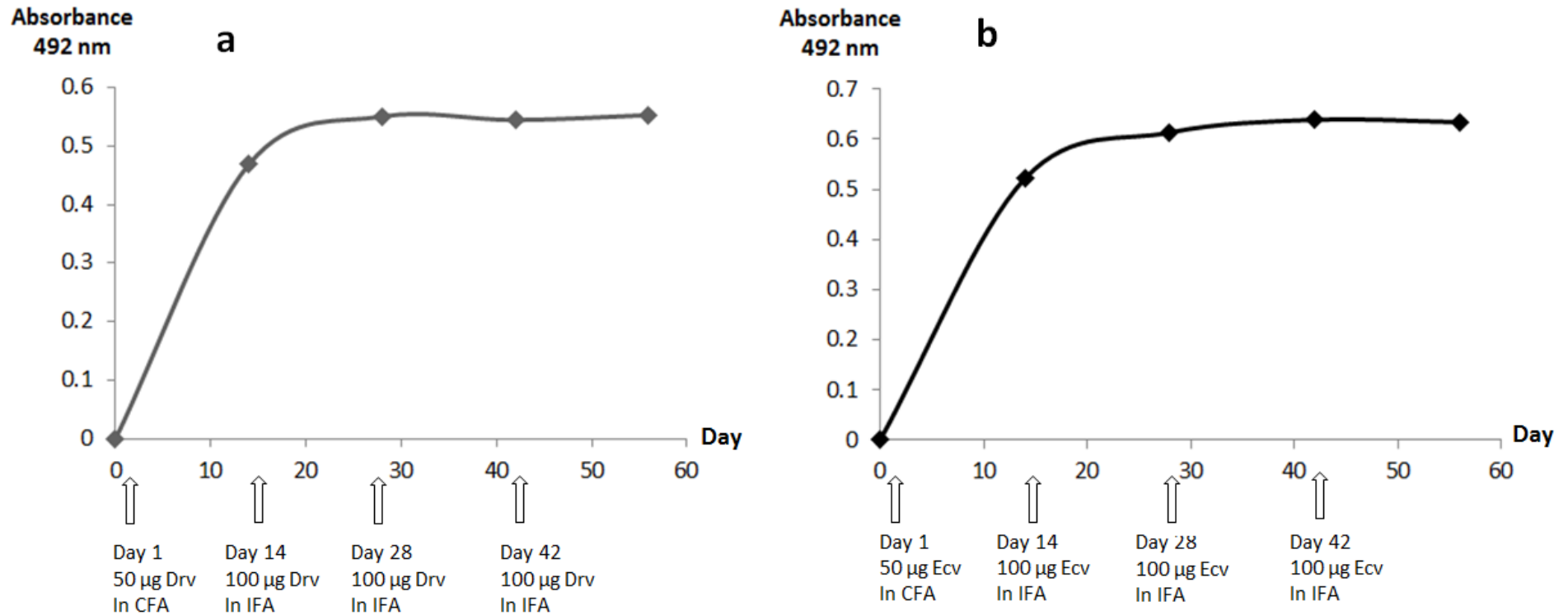


Figure 8.1 Immune responses elicited in rabbits monitored by indirect ELISA following a fortnightly immunization schedule with (a) *Sri Lankan Daboia russelii* venom, $n = 2$; (b) *Echis carinatus sinhaleyus* venom, $n = 1$. Arrows show the days of immunization with contents of injection. Drv: *D. russelii* venom; Ecv: *E. carinatus sinhaleyus* venom; CFA: Complete Freund's adjuvant; IFA: Incomplete Freund's adjuvant.

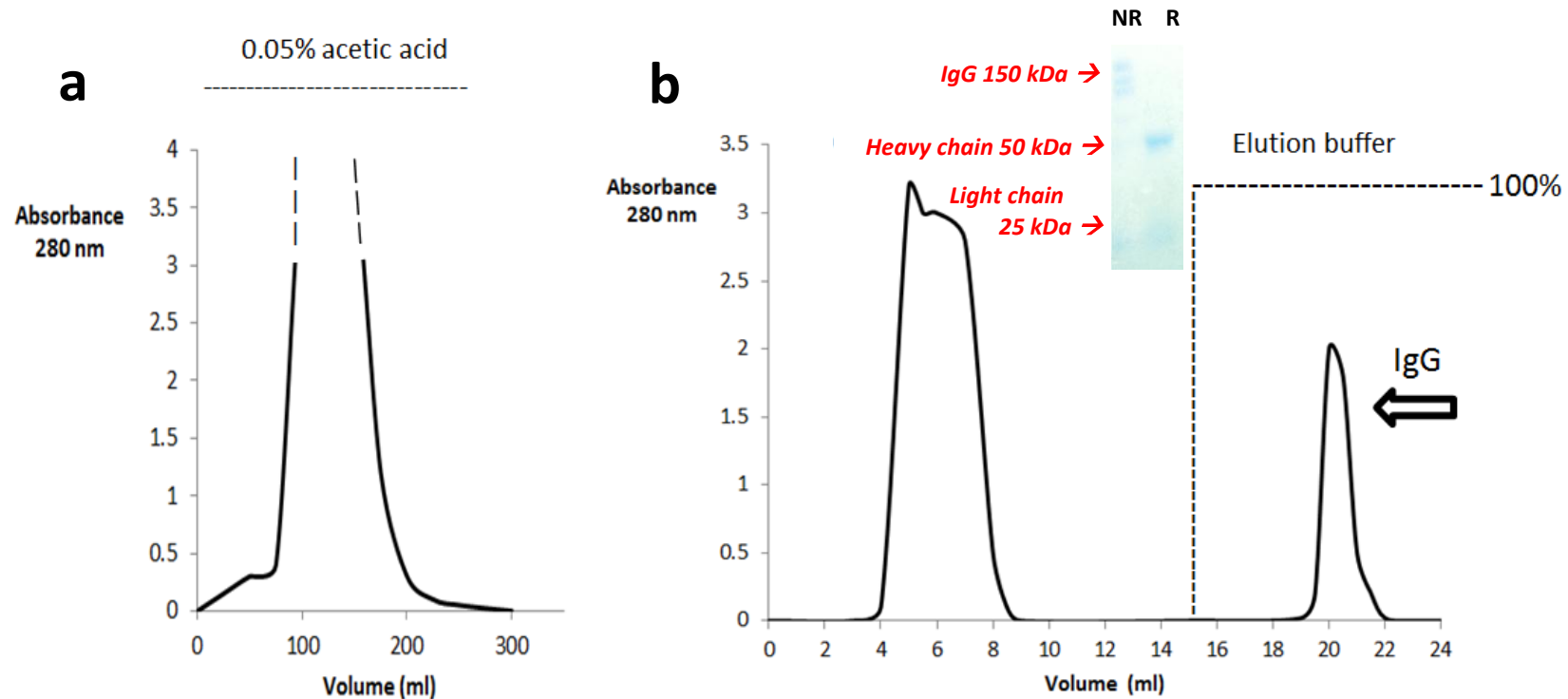


Figure 8.2 A representative diagram of step-wise isolation of immunoglobulins G (IgG) from rabbit sera: (a) Desalting of antisera using Sephadex G-25 gel filtration column, yielding serum proteins represented by one major peak; (b) Purification of IgG from serum proteins using Protein A affinity column. Arrow in (b) shows the eluted IgG fraction. Inset shows the 12.5% SDS-PAGE of isolated IgG under non-reducing (NR) and reducing (R) conditions.

8.3.3 ELISA cross-reactivity of anti-Hh sera and IgG with various venoms

Cross-reactivities of the anti-Hh antisera with various venoms on indirect ELISA were extensive: most prominent with venoms of Asiatic Crotalinae (~80-90%), moderate with viperinae (~60-80%), and least with Elapidae and Hydrophiidae (below 50%). ELISA non-specific binding by antiserum was effectively reduced by replacing the coating antisera with affinity-purified anti-Hh IgG (Table 8.1), especially on the venoms from non-Viperidae snakes. Using double-sandwich ELISA, the level of cross-reactivities of indirect ELISA was even further reduced to an extent of > 60% (Table 8.1). On both indirect and double-sandwich ELISA using affinity-purified IgG, the cross-reactivity with *C. rhodostoma* venom was consistently observed to be strongest.

8.3.4 ELISA cross-reactivities of monovalent Malayan pit viper (*C. rhodostoma*) antivenom (MPVA) and Hemato polyvalent antivenom (HPA) with *Hypnale hypnale* venom

Indirect ELISA showed that both antivenoms cross-reacted strongly with *H. hypnale* venom (> 90%) and partially with *N. naja* venom (< 50%). Using double-sandwich ELISA that has higher degree of specificity, the cross-reactivities of MPVA and HPA with *H. hypnale* venom were shown as 90.2% and 91.5% respectively, while that of *N. naja* venom was nil (Table 8.2).

Table 8.1 ELISA cross-reactivities of the anti-*Hypnale hypnale* sera and anti-*Hypnale hypnale* IgG with various venoms, using indirect or double-sandwich ELISA.

Test Venom	Indirect ELISA with antisera		Indirect ELISA with IgG		Double-sandwich ELISA with IgG	
	Cross reactivity %	± SEM %	Cross reactivity %	± SEM %	Cross reactivity %	± SEM %
Basal crotalids:						
<i>Hypnale hypnale</i>	100.0	0.0	100.0	0.0	100.0	0.0
<i>Calloselasma rhodostoma</i>	90.0	1.0	73.6	0.1	11.8	1.1
Old World, Asiatic crotalids:						
<i>Protobothrops flavoviridis</i>	91.4	1.7	42.1	1.4	10.4	0.8
<i>Protobothrops tokarensis</i>	87.1	0.2	50.9	4.3	6.5	1.0
<i>Viridovipera stejnegeri</i>	82.6	0.9	61.4	0.1	9.9	2.0
<i>Popeia popeiorum</i>	82.6	0.4	27.5	1.6	6.4	1.7
<i>Cryptelytrops. purpureomaculatus</i>	58.0	1.4	35.0	0.5	7.6	3.8
<i>Cryptelytrops albolabris</i>	76.5	0.6	40.2	1.2	2.1	2.1
<i>Tropidolaemus wagleri</i>	71.0	3.8	0.5	0.5	0.4	0.1
New World, invasion from Asia, Beringian dispersal crotalids:						
<i>Agkistrodon piscivorus leukostoma</i>	90.1	0.3	13.0	0.8	5.4	1.3
<i>Crotalus viridis</i>	83.9	2.0	38.4	0.8	7.4	1.5
<i>Crotalus atrox</i>	60.7	1.5	24.2	0.8	6.6	2.9
<i>Bothrops asper</i>	79.8	1.4	14.8	2.3	3.1	1.2

Table 8.1, continued.

Viperinae (Africa to Asia dispersal vipers)						
<i>Echis carinatus sinhaleys</i>	63.8	2.3	1.2	0.3	1.1	0.2
Viperinae (Eurasian dispersal)						
<i>Daboia russelli</i> (Sri Lanka)	80.4	2.7	2.5	0.4	2.0	0.4
<i>Daboia siamensis</i>	52.8	2.9	2.4	0.2	0.5	0.1
Elapids:						
Elapidae (Asian dispersal)						
<i>Bungarus flaviceps</i>	21.4	0.2	0.0	0.0	0.0	0.0
<i>Bungarus candidus</i>	47.1	0.5	0.0	0.0	0.0	0.0
<i>Ophiophagus hannah</i>	13.4	2.4	0.0	0.0	0.0	0.0
<i>Naja naja</i> (Sri Lanka)	13.3	2.4	0.0	0.0	0.0	0.0
<i>Naja sputarix</i> (Java)	14.9	1.1	0.0	0.0	0.0	0.0
Elapidae (African dispersal)						
<i>Naja nigricollis</i>	17.3	0.5	0.0	0.0	0.0	0.0
<i>Dendroaspis angusticeps</i>	8.5	0.7	0.0	0.0	0.0	0.0
Elapidae (Papuo-Australian)						
<i>Pseudonaja textilis</i>	7.9	0.7	0.0	0.0	0.0	0.0
Elapidae, Subfamily Hydrophiinae (Papuo-Australasian dispersal)						
<i>Oxyuranus microlepidotus</i>	7.7	0.9	0.0	0.0	0.0	0.0
Family Hydrophiidae						
<i>Enhydrina schistosa</i>	16.6	0.3	0.0	0.0	0.0	0.0

Values were expressed as mean absorbance \pm SEM. Grouping of majority of the species were done according to Wüster *et al.* (2007).

Table 8.2 ELISA cross-reactivities between monovalent MPV antivenom and Hemato polyvalent antivenom with venoms of *Calloselasma rhodostoma*, *Hypnale hypnale* and *Naja naja*, using indirect and double-sandwich ELISA.

Venoms	Indirect ELISA				Double-sandwich ELISA			
	Cross reactivity % (MPVA)	± SEM %	Cross reactivity % (HPA)	± SEM %	Cross reactivity % (MPVA)	± SEM %	Cross reactivity % (HPA)	± SEM %
<i>Calloselasma rhodostoma</i>	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
<i>Hypnale hypnale</i>	91.7	1.2	93.6	1.9	90.2	1.7	91.5	1.1
<i>Naja naja</i>	40.3	0.7	45.9	0.4	0.0	0.0	0.0	0.0

MPVA: Malayan pit viper antivenom (monovalent); HPA: Hemato polyvalent antivenom. Both antivenoms were noted effective in cross-neutralization of *H. hypnale* venom, but totally ineffective against *Naja naja* venom. *C. rhodostoma* is the homologous species from which the antivenoms were produced; *H. hypnale* is phylogenetically closest to it, while *N. naja* is phylogenetically most distant among the three.

8.3.5 Detection and quantitation of venoms in human sera

When double-sandwich ELISA was applied, the titration of *H. hypnale* venom concentrations in human serum yields a parabolic curve with a regression factor of 0.9955, within the concentration range of 0-75 ng/mL (Figure 8.3a). The assay was sensitive with the limit of detection (LoD) of 2.78 ng/mL and the limit of quantitation (LoQ) of 10.88 ng/mL venom with 1:10 dilution in human serum.

Besides, the double-sandwich ELISA demonstrated practically no cross-reactivity with the venoms of *E. carinatus sinhaleyus* and *D. russelii*, both of Sri Lankan origin. We broadened the venom identification study by including two separate assays for *D. russelii* and *E. carinatus sinhaleyus* venoms, using double sandwich ELISA as described (Figure 8.3b–8.3c). Both the assays were sensitive towards their respective homologous venoms, with the LoD and LoQ being 1.64 ng/mL and 5.75 ng/mL for *E. carinatus sinhaleyus*; while for *D. russelii*, 2.43 ng/mL and 9.83 ng/mL. Cross-reactivities of the different IgG with heterologous venoms were practically minimal and unlikely to interfere with the identification of the snake species.

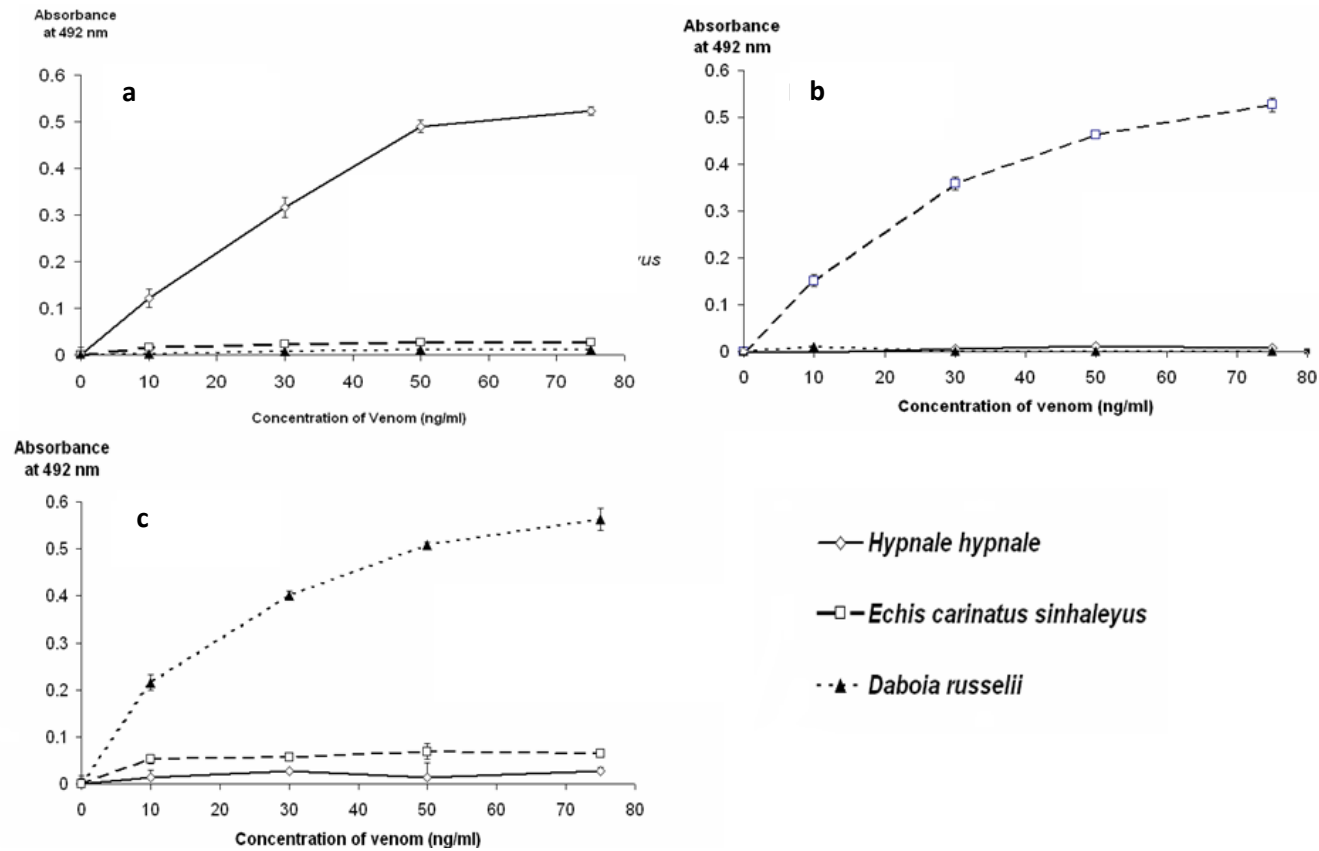


Figure 8.3 Titration curves for the concentrations of venoms from three common biting snakes in Sri Lanka dissolved in human serum, with 1:10 dilution in PBS-Tween 20, on double-sandwich ELISA using IgG for (a) *Hypnale hypnale* venom, (b) *Echis carinatus sinhaleyus* and (c) Sri Lankan *Daboia russelii*. Values were mean absorbance \pm SEM.

8.4 DISCUSSION

8.4.1 Immunization of rabbits with Sri Lankan vipers' venoms and IgG purification

The immune responses elicited in rabbits by the *Daboia russelii* and *Echis carinatus sinhaleyus* venoms, separately, were satisfactory following a fortnightly immunization schedule, and maintained with subsequent boosters. The antisera were raised against the venoms of the selected two species in order to determine the cross-reactivities of the antibodies among the three main Viperidae snakes in the region: *Hypnale hypnale*, *Daboia russelii* and *Echis carinatus sinhaleyus*, which are not uncommon in causing misidentification (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008). The study was geared towards the potential development of an immunoassay-based diagnostic tool for discerning the biting species in future clinical studies.

In pharmaceutical industry, most therapeutic antivenoms are produced by protein precipitation method, where antibody can be recovered in a relatively simple and fast way suitable for down-stream processing at large-scale basis, such as that for the MPVA and HPA. For research applications where the degree of purity and the quantity of IgG antibody are not crucial, such as in indirect ELISA and indirect flow cytometry assays, unpurified forms of antibody (as in antiserum) can be utilized. Nevertheless, purified antibodies must be used when accurate concentration or titrations are required, when chemical modifications are needed, and when non-specific binding is to be eliminated (Andrew and Titus, 2001). Several choices of antibody purification assays are available; in the current study, Protein A affinity chromatography has been employed in view of its high specificity and affinity for the IgG class from rabbit

antibodies: Protein A with its bacterial origin (from *Staphylococcus aureus*), it binds notably to mammalian IgG, disrupting opsonization and phagocytosis as an aid to the bacterium survival and, thus, virulence (Hjelm *et al.*, 1972; Bjorck and Kronvall, 1984; Goodyear and Silverman, 2003). The application in immunology research as a tool to isolate IgG antibodies has been well established (Hudson and Hay, 1980), and modified accordingly for antivenom IgG separation by various authors (Coulter *et al.*, 1980; Kukongviriyapan *et al.*, 1982; Carroll *et al.*, 1992; Selvanayagam *et al.*, 1999; Dong *et al.*, 2003; Tan, 2010; Tan *et al.*, 2012). This method produces purified IgG that is suitable for chemical modification (enzyme- or radioisotope conjugation), and to ensure minimal non-specific interaction by other serum proteins which is an important aspect in double-sandwich ELISA.

8.4.2 ELISA cross-reactivity of anti-Hh serum and IgG with various venoms

The discrepancies in the cross-reactivity readings between anti-Hh serum and anti-Hh IgG with various venoms reflected the highly non-specific nature of indirect ELISA using crude antisera, which contained various serum proteins that could have contributed to non-specific binding. Using IgG purified by affinity chromatography, false positive ELISA signals attributable to various components in the crude antisera were effectively reduced. The highest cross-reactivity of the IgG was observed with *C. rhodostoma* venom (73.6%), followed by the venoms of several Old World crotalids, with exceptionally low value for *Tropidolaemus wagleri* venom – this is not surprising as the venom is known to be quite atypical among the crotalids, whereby it does not exhibit hemorrhagic activity, is feebly procoagulant, contains unique phospholipases A₂, and is rich in atypical low molecular weight proteins (Tan and Tan, 1989; Weinstein *et al.*, 1991; Wang *et al.*, 1999b). Based on the extent of the cross-reactivities, *H.*

hypnale venom and *C. rhodostoma* venom appear to be the most similar antigenically, and this is likely due to their very close phylogenetic relatedness despite distant geographical distribution (Parkinson *et al.*, 1997). Observations where immunological properties of venom proteins supported snake taxonomy and systematics derived from mitochondrial DNA analysis had been reported in the case of *Trimeresurus* Complex (Tan, 2010; Tan *et al.*, 2012). In this study, the cross-reactivities generally became less with the increased distance of the snakes phylogenetic relationships, and were practically absent for venoms from Elapidae snakes. Generally, the cross-reactivities between the anti-Hh IgG with Crotalinae venoms appeared much stronger than that with the Viperinae and Elapidae venoms, suggesting that there are antigenically similar toxin structures conserved among the Crotalinae snakes venoms.

Double-sandwich ELISA has been known for its high specificity (Tan *et al.*, 1993) in the detection of venom antigens. It requires an antigen to be stably bound twice by a specific type of IgG: first by coating IgG, followed by secondary IgG (in this work, HRP-labeled IgG), hence it is more stringent than direct and indirect ELISA. We demonstrated that high level of cross-reactivities of indirect ELISA was reduced markedly (> 60%) using the double-sandwich ELISA, hence improving the assay specificity for *H. hypnale* venom (Table 8.1). In the case of anti-Hh IgG cross-reacting with *C. rhodostoma* venom, the different magnitudes of cross-reactivities between the indirect ELISA (73.6%) and double-sandwich ELISA (11.8%) suggest that the anti-Hh IgG, although having affinity for *C. rhodostoma* venom antigens, may have an overall lesser avidity towards them. The IgG cross-reactivities on double-sandwich ELISA were also low with the venoms of *E. carinatus sinhaleyus* and *D. russelii* (two medically important vipers in Sri Lanka), hence prompting further investigation into the potential application of double-sandwich ELISA for biting species diagnosis in *H.*

hypnale envenomation. Proper identification of that biting snake species in Sri Lanka and Southwestern India is essential as it would ensure the appropriate therapy is instituted and would this avoid the use of unnecessary antivenom that risks hypersensitivity adverse effects, especially since the biting species had been frequently misidentified in this region (Joseph *et al.*, 2007).

8.4.3 ELISA cross-reactivities of monovalent Malayan pit viper (*Calloselasma rhodostoma*) antivenom (MPVA) and Hemato polyvalent antivenom (HPA) with *Hypnale hypnale* venom

Previous studies had shown that MPVA and, to a greater extent, HPA, conferred paraspecific neutralization of *H. hypnale* venom (Chapter 5 and Chapter 6). The strong ELISA cross-reactivities of these antivenoms with *H. hypnale* venom supported the cross-neutralization reported in animal studies. In fact, the ELISA (both indirect and double-sandwich types) results have been consistent and comparable for both venoms of *H. hypnale* and, *C. rhodostoma*, the homologous species, but remarkably distinct from *N. naja* venom where no binding occurred. The findings, however, differed from the low cross-reactivity (11.8%) between anti-Hh IgG and *C. rhodostoma* venom examined on double-sandwich ELISA under the same condition (Table 8.1). This is probably due to both higher affinity and avidity of anti-*C. rhodostoma* antibodies towards the *H. hypnale* venom, as compared to that of anti-Hh IgG towards the *C. rhodostoma* venom. It is hypothesized that the anti-*C. rhodostoma* antibodies exhibit greater number of multivalencies (paratopes) that were are to recognize epitopes of *H. hypnale* venom, in contrast to anti-Hh IgG which multivalent sites are likely endowed with fewer binding sites for the antigenic epitopes of *C. rhodostoma* venom.

8.4.4 Detection and quantitation of venoms in human sera

Several factors have been known to affect the sensitivity and specificity of immunoassays (ELISA particularly) in detecting and quantitating venom antigens in biological samples - mainly, the non-specific reactivity, cross-reactivity and the quality of immuno-reagents (Ho *et al.*, 1986b). Various contributions have been made to improve the sensitivity, specificity, rapidity and simplicity of ELISA methods (Selvanayagam and Gopalakrishnakone, 1999). In the current study, the antibodies used were derived from very high titre antisera of animals after repeated immunization doses. Generally, the repeated immunization protocol hyperimmunized the animals to produce specific antibodies with high affinity as well as avidity to the immunizing antigens (Harlow and Lane, 1988) – crucial determinants for the sensitivity of immune-detection assays. Subsequent use of affinity chromatography to yield specific IgG has largely eliminated non-specific binding observed with antisera in indirect ELISA, and the specificity was further improved with double-sandwich ELISA. ELISA is known to be inherently sensitive, and since Theakston *et al.* (1977) described its use for the detection of snake venom and venom antibodies in sera, many ELISAs have been reported for snake venom detections in different types of biological samples from envenomed animals or snakebite victims (Table 8.3). Typically, the range of detection is within several nanograms of mL (ng/mL) of biological samples; and the lower the limit of detection (LoD), the higher the sensitivity of the assay. As *H. hypnale* is small in size (≤ 50 cm), the average dry mass of venom yield per milking is small too (averagely 13 mg). Given a situation where 13 mg of venoms is injected into a 60-kg Asian man with a plasma volume of 2.5 L (through bite on muscle and subcutaneous tissue), and the systemic absorption has been estimated to be 5% (unpublished data on bioavailability; see Chapter 9), the theoretical serum concentration of venom would be close to 260 ng/mL, of which 1:10 dilution would be equivalent to 26 ng/mL and is detectable and

quantifiable by the assay (LoD = 2.78 ng/mL, LoQ = 10.88 ng/mL). The sensitivity of the assay for *H. hypnale* venom nevertheless may be further improved in the future in view of the possibility of a low amount of venom injected into the victim. One suggestion of such possible optimization is the use of biotinylated antibody as shown by Selvanayagam *et al.* (1999) and Dong *et al.* (2002, 2003).

In situation where misidentification of biting species is concerned, the anti-*H. hypnale* IgG in double-sandwich ELISA demonstrated practically no cross-reactivities with the venoms of *E. carinatus* and *D. russelii*, two endemic biting snakes in the same regions which often cause misidentification for *H. hypnale*. Interestingly, anti-*E. carinatus sinhaleyus* IgG and anti-*D. russelii* IgG, apart from showing satisfactory sensitivity for its own venom antigens, demonstrated no cross-reactivities with the heterologous viperid venoms. The lack of IgG cross-reactivities among the three species venoms correlated well with the lack of cross-neutralization of the venoms (Chapter 5): *H. hypnale* venom was not cross-neutralized by Bharat polyvalent antivenom that contained anti-*D. russelii* and anti-*E. carinatus* propeptides; while Hemato polyvalent antivenom, which although neutralized *H. hypnale* venom and Sri Lankan *D. russelii* venom, was not effective against *E. carinatus sinhaleyus* venom. The result hence suggests that the double-sandwich ELISA developed from affinity-purified anti-venom IgG has the potential to be developed into a specific diagnostic kit for viperid envenomation in the region, and this is important in clinical studies where the biting species needs to be accurately diagnosed, and the blood venom levels to be monitored. It also has the potential to improve routine clinical diagnosis and to ensure that appropriate serotherapy is instituted. Although in practice, many dubious snakebite cases were empirically treated with polyvalent antivenoms, this approach can be dangerous and is not appropriate in *H. hypnale* envenomation as the polyvalent

antivenom available in Sri Lanka is only effective against *D. russelii* and *E. carinatus*, but not against *H. hypnale*. In addition, the documentation of accurate biting species identity is vital for epidemiology study, an important aspect for global health sustainability; and this also provides accurate information for future research at both clinical and laboratory settings.

In highlighting the benefits of a potential ‘Sri Lankan hematotoxic viperids’ diagnostic kit, cost is always an obstacle that should be addressed. Among the various proposed and experimental venom-screening tools, ELISA-based methods are likely the most economical, least technically demanding and most stable way as compared to radioisotopic assay, polymerase chain reaction or immunoblotting. A portable pocket-sized plate or strip can be designed to have three parallel panels, each consisting two control wells (negative and positive) and two to three sample wells for venom detection based on chromogenicity. ELISA is a rather generic facility that should be available in major medical centers in the developing countries for common serology use, and in places where such facility has been equipped, costing would mainly concern consumables like buffers, IgG, enzyme and substrate, which are generally required in very small, diluted amounts. In situations where cost and technology are permissible, the detection kit can be even further upgraded to a simplified, rapid detection version using optical immunoassay (OIA) as demonstrated by Van Dong *et al.* (2004) in Vietnam. Hence, it is hoped that with international collaborative supports, such diagnostic application can be made possible at low-resource setting in the future

Table 8.3 Enzyme-linked immunoassays (ELISA) for snake venom detection.

Snake species	Types of samples	Sensitivity (ng/mL)	Reference
<i>Bitis arietans</i> , <i>Causus maculatus</i> , <i>Echis carinatus</i> , <i>Naja haje</i> , <i>Naja nigricollis</i>	Human and rat sera	1.0	Theakston <i>et al.</i> (1977)
Australian and other snakes	Human serum, wound swab	6.0	Coulter <i>et al.</i> (1980)
<i>Bungarus candidus</i> , <i>B. fasciatus</i> , <i>Naja kaouthia</i>	Human serum	10.0-50.0	Viravan <i>et al.</i> (1986)
<i>Calloselasma rhodostoma</i> , <i>Trimeresurus albolabris</i> , <i>Daboia russelii</i> , <i>Naja kaouthia</i>	Human serum	10.0-20.0	Silamut <i>et al.</i> (1987)
<i>Vipera aspis</i>	Human blood and urine	1.0	Audebert <i>et al.</i> (1992)
<i>Calloselasma rhodostoma</i>	PBS	5.0	Tan <i>et al.</i> (1992)
<i>Bothrops</i> sp., <i>Crotalus durissus</i>	Mouse plasma	5.0	Chávez-Olórtegui <i>et al.</i> (1997)
<i>Echis carinatus</i>	Tissue homogenate	2.5	Selvanayagam <i>et al.</i> , 1999
<i>Bungarus caeruleus</i> , <i>Naja naja</i> , <i>Echis carinatus</i> , <i>Daboia russelii</i>	Tissue homogenate	0.1*	Selvanayagam <i>et al.</i> (1999)
<i>Naja atra</i>	Human sera	0.5	Huang <i>et al.</i> (2002)
<i>Trimeresurus popeorum</i> , <i>Calloselasma rhodostoma</i> , <i>Naja naja</i> , <i>Ophiophagus hannah</i>	Various biological samples	0.2-1.6*	Dong <i>et al.</i> (2003)
<i>Pseudonaja</i> sp.	Human serum	3.0	O'Leary <i>et al.</i> (2006)
<i>Oxyuranus</i> sp.	Human and rat sera	0.15*	Kulawickrama <i>et al.</i> (2010)

*with the use of biotinylated antibodies

CHAPTER 9

Pharmacokinetics of *Hypnale hypnale* Venom and Changes Effected by Hemato Polyvalent Antivenom

9.1 INTRODUCTION

Database of snake venom pharmacokinetics remains small. Studies on the pharmacokinetics of various snake venoms have not been extensively conducted, despite the usefulness of the information for a better understanding of the envenoming syndrome and for a treatment optimization. To date, there is no reported study on the time course of *H. hypnale* venom concentrations, in humans or animals. One of the reasons for this could be the lack of a specific venom quantitation assay. ELISA, an immunological assay, has the advantage in measuring blood venom concentrations as it works fundamentally on the immunological properties of the venom, the toxin proteins of which are usually the antigenic targets for antivenom to form immunocomplexes during neutralization. The measurement by ELISA hence reflects the extent of antibody-toxin binding and indicates the level of toxins in the body that correlates with the clinical syndrome.

The previous chapter (Chapter 8) describes the development of a double-sandwich ELISA using IgG specific to *H. hypnale* venom, and demonstrates the sensitivity as well as reliability of this assay in quantitating *H. hypnale* venom level in human serum. The double-sandwich ELISA was employed in this current study to monitor *H. hypnale* venom concentrations in the sera of experimentally envenomed rabbits. Changes of the venom levels in blood over time following Hemato polyvalent antivenom administration were also studied to understand the *in vivo* antivenom-venom interaction. This paraspecific antivenom was studied in this pharmacokinetic study in view of its greater neutralization efficacy and its potential use for the endemic urgency caused by *H. hypnale* envenomation (Joseph *et al.*, 2007; Ariaratname *et al.*, 2008), while there is still a long way to go before a specific antivenom can be developed.

9.2 METHODS

9.2.1 Immunization, production of anti-*Hypnale hypnale* IgG and horseradish peroxidase (HRP) conjugate

These were carried out as described in Chapter 8 to develop a double-sandwich ELISA for venom detection and quantitation (Sections 8.2.5 & 8.2.8). The IgG purification was carried out with protein A affinity column as described by Hudson and Hay (1980) with slight modification (Section 8.2.2). Conjugation of IgG with HRP was carried out according to Tijssen (1985) (Section 8.2.3).

9.2.2 Double-sandwich ELISA for venom detection and quantitation

Microtiter wells were each coated with 100 μ L of anti-*H. hypnale* (anti-*Hh* IgG (4 μ g/mL) overnight at 4 °C. After washing with 100 μ L PBS-Tween 20 for four times, the wells were incubated for 2 h at room temperature with 100 μ L of rabbit sera (1:10 dilution in PBS-Tween 20) obtained from pharmacokinetic experiments at various time intervals (Section 9.2.4). Following another cycle of washing (4 times), rabbit anti-*Hh* IgG-HRP conjugate, freshly diluted to 100 μ L (1:200 dilution in PBS-Tween 20) were dispersed into each well and incubated for another 2 h. The wells then underwent another cycle of washing (4 times), before 100 μ L of substrate (OPD) was added to initiate the enzymatic reaction. After 30 minutes the reaction was terminated by adding 50 μ L of 12.5% H₂SO₄, and the absorbance was read at 492 nm. The optimal working conditions were determined by chequerboard titration, and all assays were performed in triplicates.

9.2.3 Pharmacokinetic experiments

9.2.3.1 Envenoming model for *Hypnale hypnale* venom in rabbits

Six adult rabbits were divided into two groups: intravenous (*i.v.*, $n = 3$) and intramuscular (*i.m.*, $n = 3$) groups. Each rabbit from the *i.v.* group (1.95 ± 0.05 kg) was injected with 0.02 mg of *H. hypnale* venom in 200 μ L PBS (approximated to 0.01 mg/kg) via its marginal ear vein, whereas the rabbit from the *i.m.* group (2.03 ± 0.06 kg) was injected in the dorsal aspect of hind leg intramuscularly with 2 mg of the venom in 500 μ L PBS (approximated to 1 mg/kg). The venom dose for each route of administration was determined from preliminary studies based on the ELISA limit of measurement and the animal's tolerance to the venom toxic effect. Blood samples (1 mL) were collected serially (including pre-envenoming sample) in plain tube from the central artery of the ears. Sera were obtained by centrifuging the blood samples at 3,000 *g* for 10 minutes, and the venom concentrations at various time points were determined using double-sandwich ELISA as described above. A standard curve for venom concentrations was constructed using venom appropriately diluted in the pre-envenoming sera.

9.2.3.2 Effects of immunotherapy on the pharmacokinetics of *H. hypnale* venom

Immunotherapy for envenoming was performed with Hemato polyvalent antivenom (HPA), where 1 vial (approx. 1 g of freeze-dried F(ab')₂) was dissolved in 10 mL of normal saline. The envenoming model was the same as that described in Section 9.3.1 for the intramuscular route of venom administration: 2 mg of the venom dissolved in 500 μ L PBS was injected in the hind leg muscle. Pre-envenomed serum samples were collected from all animals prior to tests.

9.2.3.2.1 Preliminary study with a single dose of HPA

Preliminary dosing tests with 2 mL and 4 mL HPA, each given 1 h post-envenoming, were conducted. One rabbit each for the dose received the antivenom (2 mL and 4 mL, respectively) over 10 minutes via manual infusion into its marginal ear vein. Blood samples at timed intervals were collected from the central artery of the contralateral ear, and processed as described for venom concentration determination by double-sandwich ELISA (Section 8.2.5). The animals were kept in standard individual cage with access to food and water *ad libitum* throughout the experiments.

9.2.3.2.2 Repeated dosing of HPA in rabbits injected with *Hypnale hypnale* venom

A regime with two doses of 4 mL HPA, each given at 1 h and 7 h following envenoming, was adopted for 3 rabbits envenomed intramuscularly with 2 mg of the venom. The antivenom was intravenously infused over 10 minutes via the marginal ear vein. Blood samples at timed intervals were collected and processed for venom concentration determination by double-sandwich ELISA as described above (Section 9.2.3.2.1). All animals were kept in standard individual cage with access to food and water *ad libitum* throughout the experiments.

9.2.3.3 Pharmacokinetic parameters determination

Data of the serum concentration-time profiles were submitted to compartmental analysis through dissociation in exponential equations. Pharmacokinetic parameters were derived from equations described by Gibaldi and Perrier (1982) and Shargel and Yu (1999). A curvilinear relationship between the venom antigens concentrations on semilogarithmic plots and the time represents a multi-exponential equation, where the fitted curves correspond to a linear combination of two exponential terms, derived by

the method of residuals. Distribution half-life ($t_{1/2\alpha}$) and elimination half-life ($t_{1/2\beta}$) were obtained by means of the equation

$$t_{1/2} = \frac{\ln 2}{\alpha} \text{ or } \frac{\ln 2}{\beta}$$

where α and β are hybrid first-order rate constants for the distribution phase and elimination phase, respectively, obtained from the slopes of the biphasic curve on semilogarithmic plots. The individual intercompartmental rate constants k_{12} , k_{21} and the elimination rate constant k were calculated from the values obtained for α and β . The volumes of distribution (V_D), time to steady state of distribution (t_{ss}) and systemic clearance by body weight (CL) were all calculated according to Shargel and Yu (1999).

The area under the curve (AUC) was calculated using the trapezoidal rule. The concentration for 0 h was obtained by extrapolation of the concentration-time curves, and the AUCs from last determined time point (ω h) to ∞ hours was calculated as:

$$AUC_{last\ segment} = \frac{Concentration\ at\ \omega\ h}{\beta}$$

The intramuscular bioavailability ($F_{i.m.}$) of the venom was calculated according to the equation:

$$F_{i.m.} = \frac{AUC_{i.m.}}{AUC_{i.v.}} \times \frac{Dose_{i.v.}}{Dose_{i.m.}}$$

Comparison between the AUC of untreated and antivenom-treated groups was also done to assess the efficacy of the antivenom in neutralizing the venom.

9.3 RESULTS

9.3.1 Pharmacokinetics of *Hypnale hypnale* venom

Following *i.v.* administration of the venom (0.02 mg), a typical curvilinear relationship was obtained (Figure 9.1). By the method of residuals (Gibaldi and Perrier, 1982; Shargel and Yu, 1999), a biexponential equation was determined: $C_p = Ae^{-\alpha t} + Be^{-\beta t}$, where C_p is the venom level in serum at any given time, A and B are the respective y-intercepts of the distribution and elimination phases, while the α and β coefficients were derived graphically from the gradients of the biphasic curves on semilogarithmic plots. A two-compartment open model was hence adopted in this study, with descriptions made on the transfer of venom antigens between the central and tissue compartments. The time to steady state (t_{ss}) of 2.9 ± 0.9 h indicated a time point following which the venom transfer from tissue into circulation is greater than that in the distribution phase. All calculated pharmacokinetic parameters of the venom are shown in Table 9.1.

When *H. hypnale* venom (2 mg) was injected intramuscularly, the initial absorption phase demonstrated multiple peak concentrations especially in the first 30 minutes (Figure 9.2). Therefore, the absorption phase of the venom by *i.m.* route could only be explained with description for its patterns: Generally, 2 to 3 major peaks (measurable between 281-457 ng/mL) were observed within the first 30 min post-envenoming, and followed by a smaller rise (~40% height of the major peaks) by 2 h before the levels began to drop steadily. As the curve established its linear declining fashion consistent with an elimination phase, selected pharmacokinetic parameters were calculated as shown in Table 9.2. The *i.m.* bioavailability of the venom was extremely low, where only about 4% of venom injected *i.m.* was detected in the blood.

Forty eight h following venom injections, the serum venom levels were practically undetectable (< 10 ng/mL) for the intravenous profile, and were decreasing below 30 ng/mL for the intramuscular profile. The slopes of the terminal portion of the semilogarithmic graphs for each administration route appeared parallel. Figures 9.1 and 9.2 depict the venom concentration profiles on semilogarithmic plots within a time period where the best fitted linearity for terminal phase was determined.

9.3.2 Effects of antivenom on the serum pharmacokinetics of *Hypnale hypnale* venom

9.3.2.1 Preliminary study with a single dose of HPA

In a preliminary study with the *i.v.* infusion of 2 mL HPA at 1 h post-envenoming (by *i.m.* injection of 2 mg venom), the serum venom level was reduced from a peak concentration of ~200 ng/mL to ~50 ng/mL. The neutralization was largely incomplete as the venom persisted at 50-60 ng/mL in the circulation throughout the following 6 hours. This was followed by a resurged venom level to ~200 ng/mL at 7 h post-HPA infusion (Figure 9.3a). Infusion of *i.v.* 4 mL HPA at 1 h under the same envenoming condition appeared to effectively depleted the free venom antigens for the first 7 hours, but this too was subsequently followed by a rise in the venom level (to a level of ~150 ng/mL) (Figure 9.3b).

9.3.2.2 Effects of repeated HPA doses in envenomed rabbits

The intervention experiment was repeated with an initial dose of 4 mL HPA at 1 h post-envenoming, followed by an additional dose of 4 mL HPA 7 h. Immediately following the first dose, HPA effectively reduced the blood venom antigen level to < 50 ng/mL in one rabbit (80% reduction) and < 10 ng/mL in two rabbits (95% reduction) (Figure 9.4).

In one of the rabbits the blood venom level was completely depleted within an hour following the first dose of HPA, while a small increment of venom antigen level to ~50 ng/mL was noted in the other two rabbits following the initial drop. Nevertheless, the venom levels continued to drop markedly and were soon depleted in one of the two rabbits; while in the last rabbit, the venom level decreased further to ~10 ng/mL towards 7 h before the second dose of HPA was administered. The resurgence of venom antigens anticipated in the later course of envenoming (Figure 9.3a-b) was prevented by the second dose of HPA (Figure 9.4). Overall, the mean concentrations of venom antigens at various time points following HPA administrations at 1 h and 7 h were significantly lower than those in the untreated group ($p < 0.05$, Student's t-test) (Figure 9.5a-b). In addition, the total areas under the curve (AUC) representing the total concentrations of venom in blood (Figure 9.5b) for the treated group (494.6 ± 174.4 ng·h/mL) was significantly lower when compared to that of the untreated group (5511.7 ± 1291.5 ng·h/mL) ($p < 0.05$, Student's t-test). For visual and comparative purpose, Figure 9.4 depicts the time course profiles of individual treated rabbits, while Figure 9.5a and Figure 9.5b show the concentration-time profiles (based on mean values) on normal and semilogarithmic plots, respectively.

Venom concentration (ng/mL)

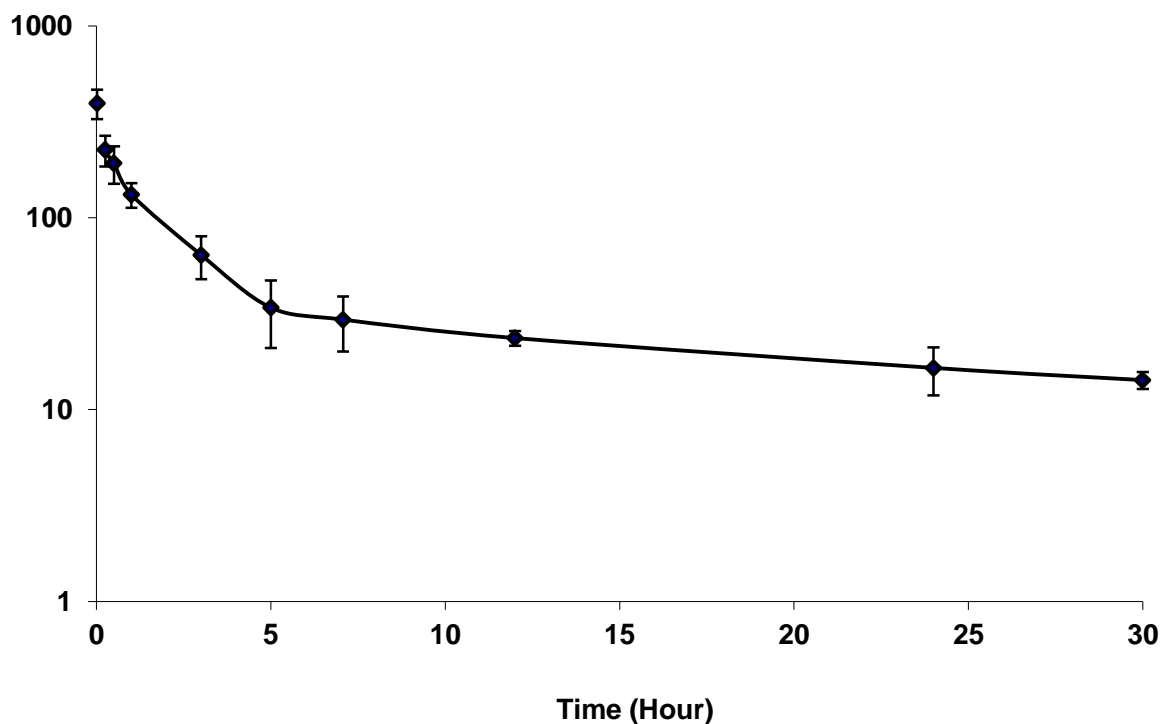


Figure 9.1 Pharmacokinetic profiles of *Hynale hynale* venom (0.02 mg) injected intravenously on semilogarithmic plots. Concentrations of free venoms in the sera were measured with double-sandwich ELISA. Values are the means \pm S.E.M. of three independent experiments using a rabbit model.

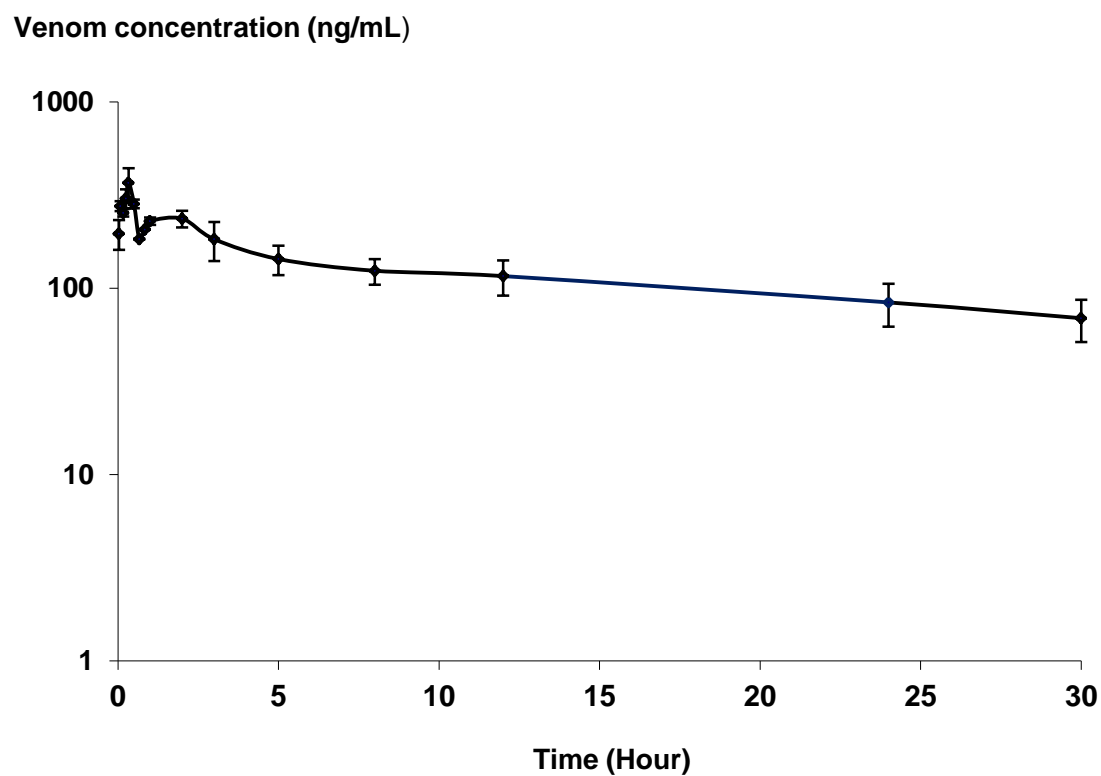


Figure 9.2 Pharmacokinetic profiles of *Hypnale hypnale* venom (2 mg) injected intramuscularly on semilogarithmic plots. Concentrations of free venoms in the sera were measured with double-sandwich ELISA. Values are the means \pm S.E.M. of three independent experiments in a rabbit model.

Table 9.1 Pharmacokinetic parameters of *Hypnale hypnale* venom when injected intravenously in rabbits (Injection dose = 0.02 mg venom in 200 μ L PBS).

Parameter	Description	Unit	Mean	S.E.M.
C_0	Concentration at t_0	ng/mL	346.85	68.94
α	Rate constant for distribution phase	h^{-1}	1.39	0.27
β	Rate constant for elimination phase	h^{-1}	0.04	0.00
$t_{1/2\alpha}$	Half-life at distribution phase	h	0.54	0.10
$t_{1/2\beta}$	Half-life at elimination phase	h	20.10	2.45
V_c	Volume of central compartment	mL	62.49	12.39
$(V_D)_{ss}$	Apparent volume of distribution at steady state	mL	329.62	30.51
$(V_D)_{area}$	Volume of distribution by area	mL	390.73	75.21
CL	Systemic clearance by body weight	mL/h/kg	7.45	1.51
t_{ss}	Time at steady state	h	2.83	0.50
$AUC_{0-\infty h}$	Area under curve from time zero to infinity	ng·h/mL	1441.9	244.67

Table 9.2 Pharmacokinetic parameters of *Hypnale hypnale* venom when injected intramuscularly in rabbits (Injection dose = 2 mg venom in 500 μ L PBS).

Parameter	Description	Unit	Mean	S.E.M.
β	Rate constant of elimination phase	h^{-1}	0.04	0.00
$t_{1/2\beta}$	Half-life at elimination phase	h	19.31	0.73
$(V_D)_{\text{area}}$	Volume of distribution by area	mL	405.03	65.69
CL	Systemic clearance by body weight	mL/h/kg	7.38	1.50
$\text{AUC}_{0-\infty\text{h}}$	Area under curve from time zero to infinity	ng·h/mL	5511.71	1291.49

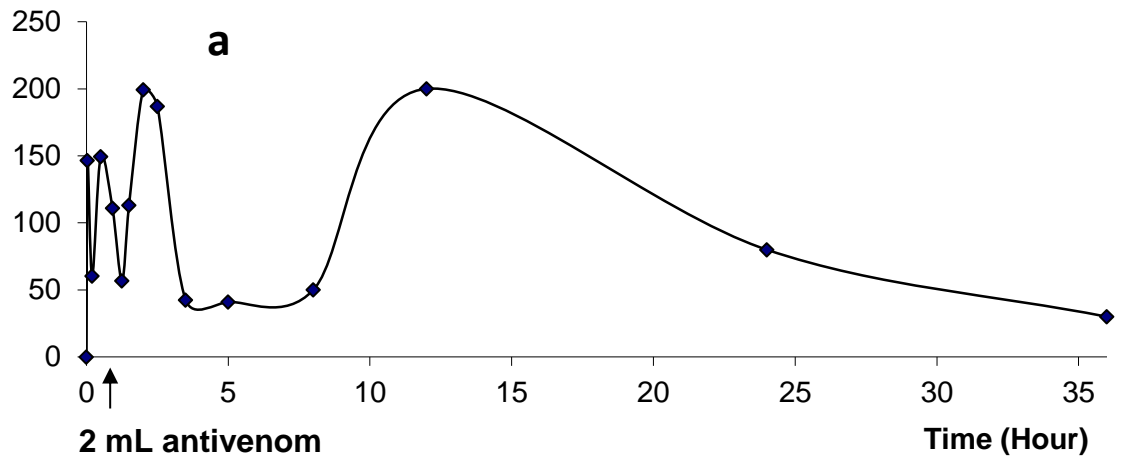
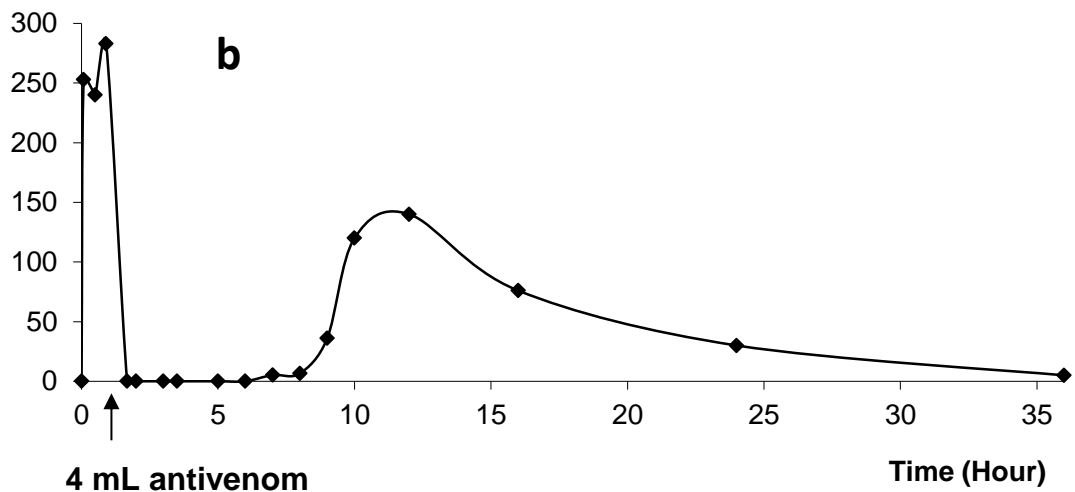
Venom concentration (ng/mL)**Venom concentration (ng/mL)**

Figure 9.3 Modifications of pharmacokinetics of 2 mg intramuscularly injected *Hypnale hypnale* venom in rabbits induced by (a) 2 mL, and (b) 4 mL of Hemato polyvalent antivenom (HPA) administered 1 h post-enuenomation. Values are the venom concentration in blood at various time points in each rabbit. The venom concentrations were labeled on a normal axis to demonstrate the resurgence in blood venom concentrations.

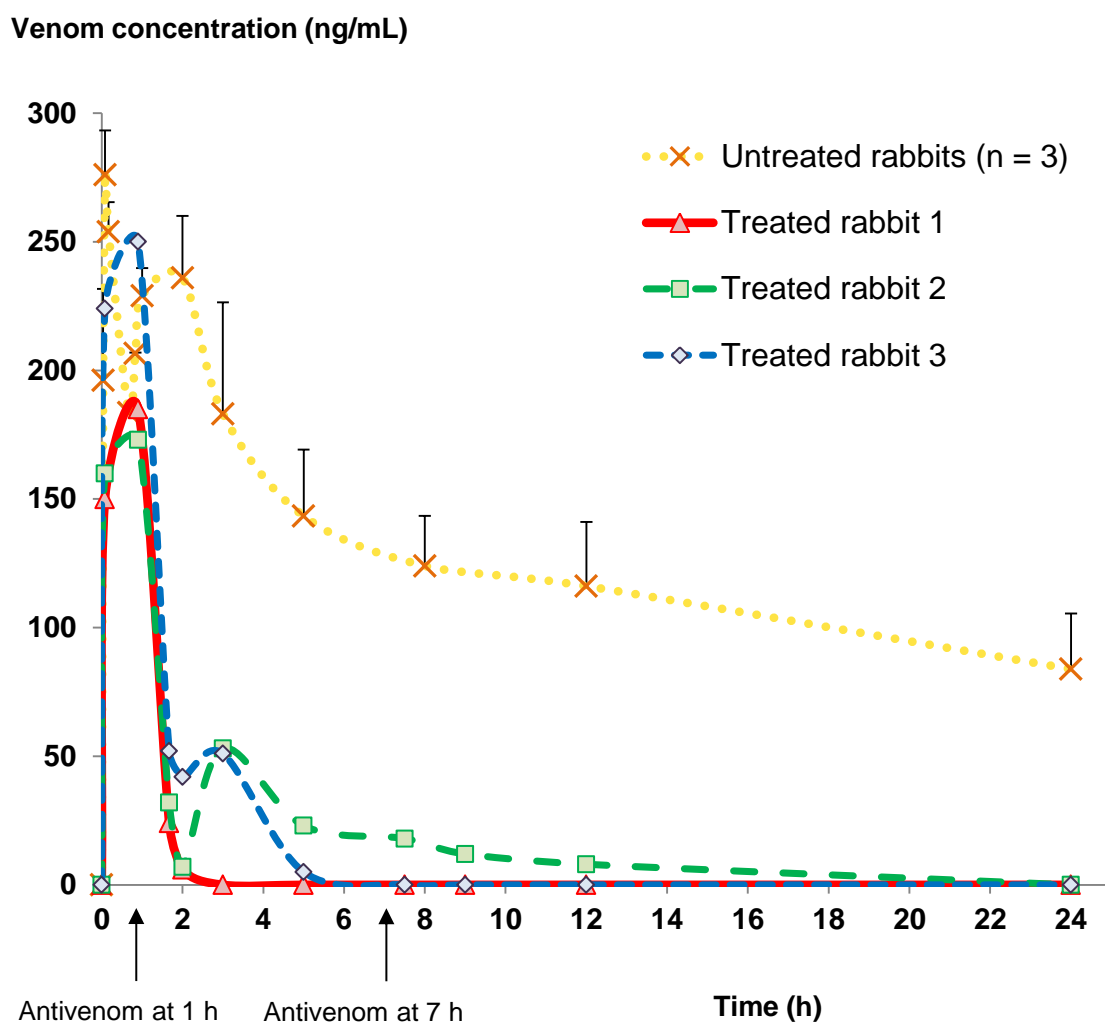


Figure 9.4 Effect of Hemato polyvalent antivenom (HPA, constituting a “4+4 regimen”) on the serum pharmacokinetics of *i.m. Hypnale hypnale* venom in 3 rabbits which time course profiles were illustrated individually. Included on the plots was the mean concentrations \pm S.E.M. for an envenomed group of rabbits without antivenom treatment. All venom concentrations were labeled on a normal axis to demonstrate the resurgence in blood venom concentrations.

(Figure 9.5)

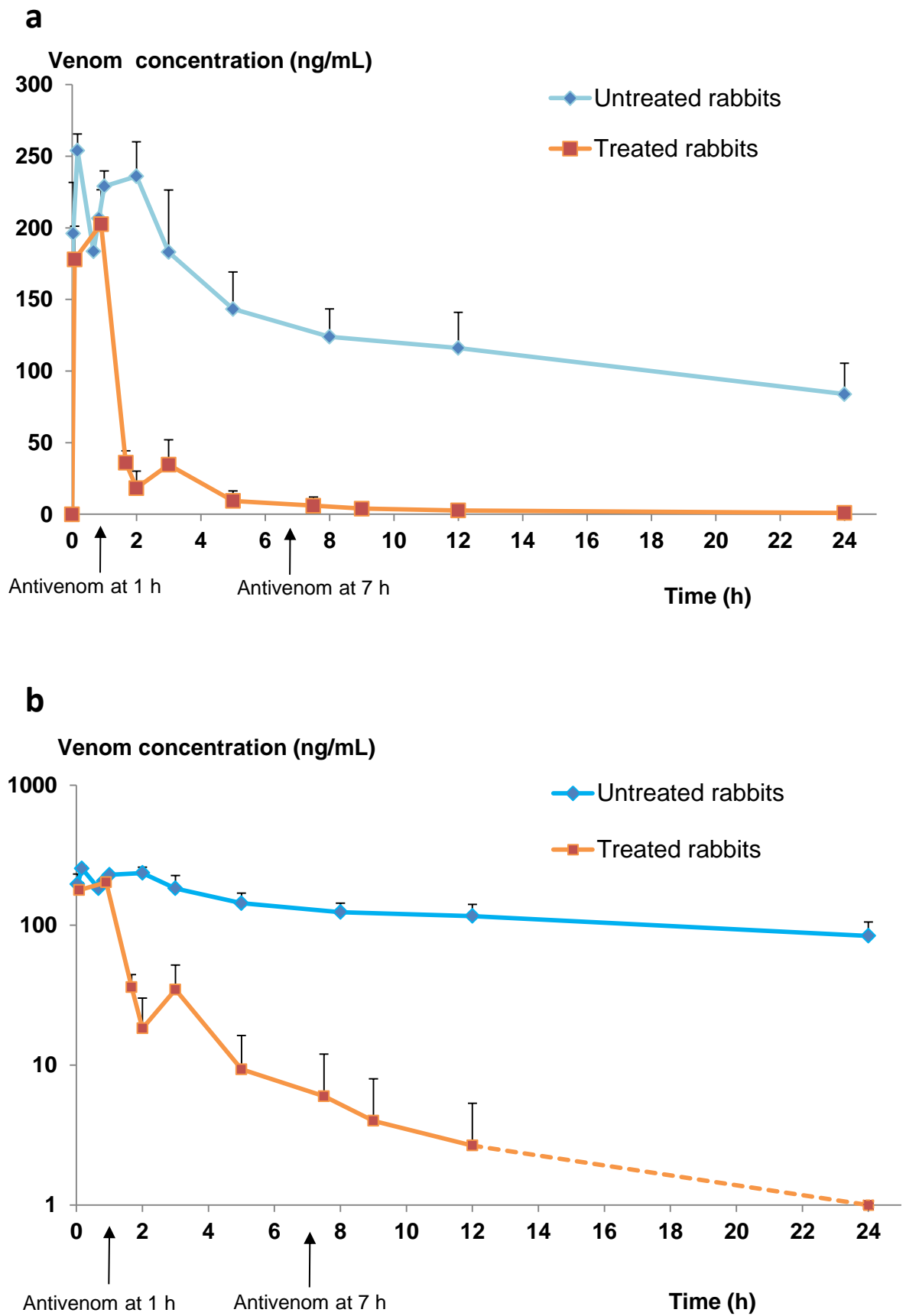


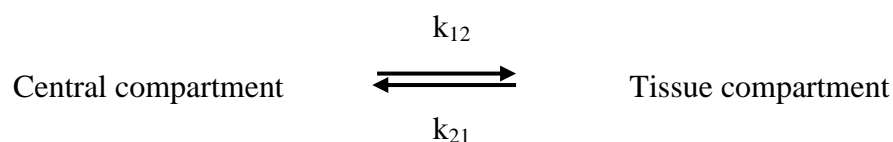
Figure 9.5 Effect of Hemato polyvalent antivenom (HPA, constituting a “4+4 regimen”) on the serum pharmacokinetics of *i.m. Hypnale hypnale* venom. Rabbits (2 kg) were envenomed intramuscularly with 2 mg venom with and without subsequent antivenom treatment. Blood venom antigen levels were shown on (a) normal plots and (b) semilogarithmic plots. In (b), dashed line (from 12 h to 24 h) shows an imaginary line representing the decline in blood venom concentrations by 24 h (which could have possibly completed earlier by then, between 12-24 h). Arrows show the time points at which 4 mL of antivenom was infused. Values are the means \pm S.E.M. of three independent experiments for each antivenom-treated and untreated group.

9.4 DISCUSSION

9.4.1 Pharmacokinetics of *Hypnale hypnale* venom

Depending on the routes of administration (*i.v.* or *i.m.*), different doses of venom were given to the animals. The venom dose for intramuscular injection was higher than that injected intravenously to compensate for the venom's low bioavailability in order to yield blood venom concentrations that were within a meaningful quantifiable range. The low *i.v.* dose when given intramuscularly resulted in very low sera concentrations way below the range of ng/mL that does not reflect the actual situation of most snakebite envenomations. The *i.m.* dose was therefore increased accordingly by trials to 2 mg and it was with this amount that the venom concentrations were measured reliably and comparable to the intravenous group (within a concentration range of 10-1000 ng/mL). An attempt to standardize the *i.v.* dose based on the relatively higher *i.m.* dose unfortunately appeared lethal, almost instantly, to the animal. The phenomenon was likely due to the procoagulant nature of the venom that precipitated an acute thromboembolic event by direct intravenous route. Hence, this explains the difference in venom dosages administered by different route in this study. During data analysis, pharmacokinetic parameters dependent on the initial dosage were normalized by the ratio between intravenous and intramuscular doses.

Following intravenous injection, the pharmacokinetics of *H. hypnale* venom followed a two compartmental open model as depicted below:



Symbols k_{12} and k_{21} represent the intercompartmental transfer rate constants, respectively, from the central compartment to the tissue compartment and vice versa. The venom exhibited a fast distribution from the central (typified by the vascular compartment and highly perfused organs) to the peripheral (tissue) compartment, evidenced by a relatively short distribution half-life ($t_{1/2\alpha}$), and a greater transfer into the tissues with a k_{12}/k_{21} ratio of 3.2. This finding of fast distribution is comparable to that of *Vipera aspis* (a European viper) venom by intravenous route (~ 0.7 h); while its elimination half-life ($t_{1/2\beta}$) was considerably longer than that of *V. aspis* venom (~ 12 h), studied in rabbits (Audebert *et al.*, 1994). Similar patterns of a fast distribution ($t_{1/2\alpha} < 0.5$ h) followed by a slow elimination ($t_{1/2\beta} < 10$ h) has been reported for the viperid venoms of *Bothrops erythromelas* ($t_{1/2\alpha} \sim 0.2$ h, $t_{1/2\beta} \sim 6.6$ h; Rocha *et al.*, 2008) and *Vipera russelli* (*Daboia russelii*) ($t_{1/2\alpha} \sim 0.3$ h, $t_{1/2\beta} \sim 8.9$ h; Maung-Maung-Twin *et al.*, 1988), both studied in mice. Although venoms of the Viperidae family is known to generally contain similar high molecular mass enzymatic proteins and hence may exhibit similar distribution and elimination patterns, it is noteworthy to mention that experiments in mice might generate smaller parameter values for V_D and $t_{1/2}$ due to the low body weights and large body surface area to mass ratios in these animals. The *H. hypnale* venom appeared readily distributed into the tissue compartment based on its large volume of distribution by area, $(V_D)_{\text{area}}$, which is approximately three times of a 2-kg rabbit's total blood volume (120-150 mL). The high affinity towards tissues and low systemic clearance by body weight (CL) of this venom are likely accountable for its long $t_{1/2\beta}$ observed in this study. Clinically, this indicates that the venom systemic effects could be sustained well over many days, consistent with the prolonged or late onset clinical features typically related to consumptive coagulopathy (Ariaratnam *et al.*, 2008; Sunanda *et al.*, 2010). In view of the venom slow elimination and prolonged or late complications, antivenom treatment seems to have a favorable therapeutic outcome

as it may still benefit patients who present late to the healthcare before the complications set far.

On the intramuscular serum kinetic profiles, each concentration peak during the short absorption phase possibly represented the C_{\max} (maximal concentration) of one or collectively several toxin components from the venom, due to their varied initial rates of absorption from the perivascular injection site (Tan *et al.*, 2009). Nevertheless, the $t_{1/2\beta}$, $(V_D)_{\text{area}}$ and CL of *H. hypnale* venom intramuscularly injected were all comparable to that of the intravenous profile, reflecting the consistency of the venom systemic pharmacokinetic behavior. The similarity between the *i.v.* and *i.m.* $t_{1/2\beta}$ for *H. hypnale* venom indicated that the slow elimination process is likely the rate-limiting step for its terminal phase, although a “sustained release” of venom from the *i.m.* injection site could be a concurrent contributing cause of prolonged terminal half-life, as reported by Auderbert *et al.* (1994).

The very low intramuscular bioavailability of *H. hypnale* venom, on the other hand, implies poor systemic absorption of the venom from the injection site. From the literature search, it was found only one article where *i.m.* bioavailability of whole snake venom was reported (67% for *Vipera aspis* venom, Auderbert *et al.*, 1994). General pharmacokinetic concept would predict a nearly 100% bioavailability for most therapeutic agents given intramuscularly, although exception has been reported for $F(ab')_2$ antivenom which *i.m.* bioavailability was as low as 36% (Pépin-Covatta *et al.*, 1996), possibly due to the large molecular size of the protein. Contrary to the pharmacokinetic concept, existing data showed that at least two viperid venoms were not completely absorbed via the intramuscular route. Possible explanations for this anomaly include:

- (1) Impeded absorption due to major local tissue destruction and compromised vasculatures (see Chapter 4), especially for the abundantly high molecular mass venom proteins;
- (2) Persistent binding of some venom components at the injection site, followed by *in situ* degradation as local inflammation and necrosis set in, triggering phagocytosis and destruction of the toxins before they were circulated;
- (3) Altered structures of the venom antigen epitopes following interaction with local tissues, which in turn affects the ELISA detectability.

The phenomenon illustrates a ‘pre-systemic loss’ for *i.m.* venom absorption that is likely a result from the local toxicity of the venom itself. For the first time, we try to explain the variation between *i.v.* and *i.m.* LD₅₀ of venom by correlating lethality to bioavailability: a huge difference of fifteen times between the *i.v.* and *i.m.* LD₅₀ of *H. hypnale* venom (0.90 and 13.7 µg/g, respectively, Chapter 4) could be accounted for by the venom *i.m.* bioavailability of less than 10%. In the case of *Vipera aspis* venom, the *i.v.* LD₅₀ was reported as 1.0 mg/kg by Brown (1973) and 0.55 µg/g by Komori *et al.* (1998); while Tu *et al.* (1969) reported 4.7 mg/kg for its *i.m.* LD₅₀. The LD₅₀ varied by five to ten times between the two routes of administration, although the *i.m.* bioavailability reported by Auderbert *et al.* (1994) appeared higher (67%) by proportion. Further interpretation of this phenomenon is however limited as the LD₅₀ values were derived from rodent models, which were different from rabbits in most pharmacokinetics studies. On the other hand, the very low systemic absorption of *H. hypnale* venom by *i.m.* route is in accord with clinical case reports where local envenoming represented the commonest presentation of *H. hypnale* bites (91%), in contrast to systemic envenoming (39%) characterized by hemostatic disturbances

(Ariaratnam *et al.*, 2008). The venom however appears highly toxic and potentially fatal should it enter the circulation, with its systemic effect being predominantly “hematoxic” (causing deranged hemostasis and coagulopathy). Acute kidney injury – a severe though infrequent fatal consequence has been suggested as a complication of disseminated intravascular coagulopathy, mediated through the procoagulant and fibrinolytic actions of *H. hypnale* venom (Kanjanaabuch and Sitprija, 2008; Sitprija, 2008). Therefore, in view of the venom’s long elimination half-life and its prolonged hematoxic nature in general, the coagulation profile as well as renal function of patients should be monitored closely on an extended basis over days and even weeks, as some disastrous bleeding complications might present as late as 5 weeks post-bite (Sunanda *et al.*, 2010).

9.4.2 Effects of antivenom on the serum pharmacokinetics of *Hypnale hypnale* venom

9.4.2.1 Preliminary dosing

Preliminary dosing with 2 mL HPA given 1 h post-envenoming was insufficient for complete neutralization of the venom. The subsequent rise in the venom level several hours later presumably was caused by an enhanced transfer of venom antigens from tissues into the vascular space. These redistributed venom antigens, as expected, were beyond the neutralizing capacity of the 2 mL HPA administered several hours ago.

The intervention repeated with 4 mL HPA at hour 1 effectively reduced the free venom antigens for the following 7 hours. This reflects that a higher first dose of antivenom is needed to neutralize the circulating venom toxins following envenomation. A drastic drop in plasma free venom level induced by *i.v.* antivenom infusion has been reported, and the phenomenon is vital since the plasma venom level correlates well with the

severity of envenomation (Ismail *et al.*, 1996; Otero *et al.*, 1996). This implies that the amount of first dose antivenom sufficient to optimally reduce the initial venom level should be determined during clinical study in order to preempt the onset of severe envenoming. In fact, the initial dosages of most antivenoms recommended for use in snakebites in South Asia/Southeast Asia are within 5 to 10 vials, considerably high doses to ensure early and adequate elimination of the venom (WHO, 2010). Following that, antivenom can be repeated empirically as when indicated clinically (by incomplete resolution of systemic effects). However, recovery may be delayed on blood coagulability even when a large dose of antivenom is given to adequately neutralize the circulating venom procoagulant enzymes. This is due to the fact that generally 3-9 hours are needed for the liver to restore coagulable levels of fibrinogens and other clotting factors (WHO, 2010). Nevertheless, an early and adequate antivenom treatment is principally the aim of antivenom therapy to ensure an early elimination of venom from the system for a faster recovery before complications set far.

The single dose of 4 mL HPA given at 1 h, unfortunately, did not prevent the venom resurgence in blood that occurred ~7 h after its administration. The venom resurgence however appeared slightly smaller than that observed in the case of 2 mL HPA, although in essence, it reflects the inadequacy of the antivenom to sequester the subsequently resurged venom antigens. This could be due to either an insufficient amount of functional paratopes, or a lack of affinity towards certain toxin antigens that reappeared into the blood.

9.4.2.2 Venom redistribution and repeated dosing of HPA

A rebound increase of drug concentration in blood following immunotherapy has been described for drugs like digitoxin (Smith *et al.*, 1976) and colchicine (Sabouraud *et al.*,

1992) in overdose poisoning. The term ‘drug redistribution’ has been coined to denote the enhanced transfer of a drug from tissue into vascular compartment following immunotherapy for the drug. Similar phenomenon on venom ‘redistribution’ induced by antivenom had also been reported, both in snakebites (Rivière *et al.*, 1997; Rocha *et al.*, 2008) and scorpion stings (Revelo *et al.*, 1996; Krifi *et al.*, 2005). The recurrent venom antigenemia occurs mainly by following the law of mass distribution (Chippaux, 2006), where the shifted intercompartmental equilibrium (due to clearing of circulating venom antigens within a brief period) favors the transfer of tissue-deposited antigens into the blood. Such modification of venom pharmacokinetics depends not only on the affinity of antibodies for toxins but also on the dose of antivenom and the timing in antivenom administration (Rivière *et al.*, 1997; Gutiérrez *et al.*, 2003). The redistribution phenomenon however provides a mechanism essential for detoxification as it extracts venom toxins deposited in the peripheral tissues into the blood, where the redistributed toxins can be further bound by circulating antivenom for elimination (Gutiérrez *et al.*, 2003, 2007). It is also known that for viperid venoms that cause local tissue destruction (due to hemorrhagic and necrotizing toxins e.g. metalloproteases), antibody-induced redistribution can halt further tissue damages, although it is unlikely to restore the already affected areas with irreversible damages (Gutiérrez *et al.*, 1998).

In anticipation of a venom redistribution phenomenon induced by the first dose of antivenom, an additional dose of HPA was administered at 7 h post-envenoming. The efficacy of the first dose of HPA in producing a drastic reduction (80-90%) of the venom levels in all rabbits indicated that the antivenom is effective against the major toxins in the heterologous venom, although minor variations in the decline of venom level were initially observed in two rabbits. Nevertheless, the reduction was still statistically highly significant compared to the untreated group. The subsequent dose of

HPA enhanced the clearing of residual venom antigens in one rabbit, evidenced by the steeper gradient on its terminal phase. More importantly, however, this additional dose of HPA successfully prevented the anticipated venom redistribution in all rabbits. The extent of immunocomplexation (the principal therapeutic mechanism of antivenom) between the HPA doses and the major toxins was excellent, as indicated by the significantly smaller AUC in the treated group.

The minor variations noted in the declining pattern of venom level following the first dose of HPA, based on an *in vivo* experiment as such, appear to imply the following:

- (a) Variations in pharmacogenetics of the animals could have affected the antivenom metabolism and consequently its interactions with the venom antigens;
- (b) The time for administering the first dose of antivenom (1 h post-envenoming) was when the venom's complex absorption and distribution largely took place (from the time of envenoming up to 3-5 h), and thus complicating the neutralizing effect;
- (b) Certain antigens were not optimally bound by HPA at the dose of 4 mL. The HPA is, after all, a paraspecific antivenom; whereas the ELISA was developed using anti-*H. hypnale* venom IgG that could have detected specific antigens 'missed' by HPA in the circulation. A much higher dose of HPA (> 4 mL), nonetheless, may be possible to overcome such shortcoming in view of the paraspecific origin of the antivenom, and hence representing a potential future work for dosing optimization.

Essentially, the significant pharmacokinetic changes indicate a remarkable therapeutic potential with the use of HPA for *H. hypnale* envenoming. In fact, the neutralization of venom antigens, characterized as a graded response, does not necessarily work on an 'all-or-none' (none being zero antigen level) basis: an antivenom with good affinity can

significantly reduce the venom concentration to a subtoxic level, thereby preventing or reversing the envenoming syndrome. In the current pharmacokinetic study, the reduction and subsequent complete depletion of *H. hypnale* venom antigens by HPA is consistent with previous pharmacodynamic outcomes where HPA has been proven to neutralize the lethal, hemorrhagic, necrotic, procoagulant, as well as nephrotoxic effects of the venom (Chapter 5 and chapter 6). The findings hence imply that the “4+4 regimen” of HPA appears to be timely and adequate to assure satisfactory immunocomplexation with the circulating venom toxins, as well as promoting venom redistribution from tissues into vascular space where further neutralization takes place.

Interestingly, although the *in vitro* neutralization test showed that 1 mL of HPA could neutralize 1.5 mg of *H. hypnale* venom (based on ED₅₀ calculation, Chapter 5, see Table 5.1), a total of 8 mL HPA was required in current *in vivo* pharmacokinetic experiment (where 2 mg of venom was injected intramuscularly in rabbit) for the venom antigens to be effectively neutralized over the study period. Such observation based on an *in vivo* animal model suggests that an initial antivenom dose higher than that established *in vitro*, coupled with repeated dosing, may be necessary for adequate *in vivo* venom neutralization over the prolonged time course. It also emphasizes the fact that *in vitro* neutralization tests should always be supported by *in vivo* pharmacokinetic experiments for a more realistic and practical understanding of the pharmacology of an antivenom, as the venom (and the corresponding antivenom) introduced/administered into a biological system is bound to undergo pharmacokinetic processes, specifically, distribution and elimination. These deductions based on experimental basis are potentially useful as complementary information for treatment design. Clinical trials are therefore required in the future to establish an optimized protocol of the antivenom treatment.

CHAPTER 10

Proteomic Study of *Hypnale hypnale* Venom

10.1 INTRODUCTION

Pit vipers (Crotalinae) are the most widely distributed subfamily of the Viperidae family, with major radiations over 190 species (~75% of viperid species allocated in 29 genera) (<http://reptile-database.org>) in both the Old and New Worlds. Over millions of years the diverse ecological niche has triggered off accelerated evolution, characterized by rapid amino acid sequence divergence of venom proteins that led to a tremendously diverse number of proteins and peptides in snake venom with equally diverse biological functions. Knowledge of the diversity of venom proteins entails identification of various proteins/peptides in the venom of a particular species. Although with current molecular technology, transcriptomics of venom gland virtually unmask the identity of all proteins/peptides at transcript level, it does not provide quantitative data on their relative amount according to various post-translational proteins i.e. the composition of toxins. Venom proteomic studies nevertheless address the concern by providing information on the types and composition of major toxins in a venom; hence, a better understanding of the pathophysiology of the venom. This is especially relevant for medically important species which still lacks characterizations of the venom properties, e.g. *Hypnale hypnale*. Unfortunately, there can be no single unique method utilized for all proteomic analyses to date. In the study of *H. hypnale* venom proteome, two approaches were adopted: (1) trypsin-digested venom subjected to shotgun-LC-MS/MS; (2) venom separated by reversed-phase HPLC and SDS-PAGE, followed by N-terminal amino acid sequencing and protein digestion for peptide mass fingerprinting using tandem mass spectrometry. An additional effort of de novo peptide sequencing was undertaken for sequences not matched to current database, likely due to a lack of species-specific transcriptomic database.

10.2 Methods

10.2.1 Shotgun-LC-MS/MS and *de novo* sequencing of *Hypnale hypnale* venom proteins

The venom protein sample (1 mg) was reduced, alkylated and trypsin digested according to the iTRAQTM Reagents protocol (Applied Biosystems Sciex). Peptides were analysed by LC-MALDI analysis using the UltiMateTM 3000 nano-HPLC system (Dionex, C18 PepMap100, 3 µm) equipped with a ProBot robotic spotter (LC Packings) coupled to AB SCIEX TOF/TOFTM 5800 System Proteomics Analyzer (Applied Biosystem Sciex). In brief, peptides were loaded onto the reverse-phase HPLC and separated with a gradient of 10-45% acetonitrile (containing 0.1% trifluoroacetic acid) over 165 minutes. Spectra were analysed to identify protein of interest using Mascot sequence matching software (Matrix Science) with Uniprot database and taxonomy set to Serpentes (Downloaded September 2011; 15,468 sequences).

For *de novo* sequencing analysis, the venom sample was resuspended in 100 µL of 2% CH₃CN/H₂O (containing 0.1% TFA). From this solution 2 µL was added to 18 µL of 2% CH₃CN/H₂O (containing 0.1% TFA). Ten µL was injected onto the UltiMateTM 3000 nano-HPLC system (Dionex, C18 PepMap100, 3 µm) and separated with a gradient of 10-40% acetonitrile (containing 0.1% TFA) with spotting using a ProBot robotic spotter (LC Packings) over 180 min. Peptides were analysed by MALDI-TOF-TOF mass spectrometer using AB SCIEX TOF/TOFTM 5800 System Proteomics Analyzer (Applied Biosystem Sciex). MS/MS spectra were analysed using PEAKS Studio Version 4.5 SP2 (Bioinformatics Solutions). All *de novo*-derived sequences from MS/MS spectra were submitted for BLAST (Basic Local Alignment Search Tool)

search in the *blastp* suite at www.ncbi.nlm.nih.gov/BLAST, using the following settings for the non-redundant protein sequences (nr) database:

Taxonomy = Serpentes (taxid: 8570) for Sequence 1-10, except Sequence 4 with
Viperidae (taxid: 8689).

Expect threshold = 10

Word size = 3

Matrix: BLOSUM 80

Gap costs: Existence: 10 Extension: 1

Compositional adjustment: No adjustment

Filter: not selected

Following BLAST searching, the *de novo*-derived sequences were putatively assigned a protein family and a function using a scoring system modified from Bringans *et al.* (2008). In brief, for each peptide, the functions of the homologous BLAST hits with the three highest hit scores (generally correlated with the lowest three Expectation values/E-values) were evaluated from the database entry. A score of one point was given to a functional category each time the function is matched to a protein within the top three hits. For matches with equivalent hit scores and E-values, proteins of the same family (note the different accession numbers though) but originated from different species were treated as different entities considering the extreme diversity of venom proteins; hence, one point would be scored for each match. However, a maximum of one point only was given to matches with equivalent hit score or E-value if the particular protein originated from a same species; or if the matches reflected sequence repetition within a protein molecule. The *de novo*-derived sequence is then putatively assigned the functional protein category with the highest overall score. Where two or more functions had

equivalent scores, the function would be labelled as undetermined. Sequences assigned to a same protein family were checked for the accession numbers and organism sources of the scoring proteins to determine if they possibly belong to the same protein molecule.

Shotgun-LC-MS/MS and *de novo* sequencing were conducted at Proteomics International, Perth, Australia which provided the service on mass spectrometry proteomic facility. Data analysis and BLAST search of the *de novo*-sequences were completed back in the University of Malaya, Kuala Lumpur, Malaysia.

10.2.2 Reverse-phase HPLC separation of *H. hypnale* venom proteins and further characterizations

Two milligrams of *H. hypnale* venom dissolved in 0.1% TFA was fractionated on a LiChrospher[®] RP100 C18 reverse phase column (Merck, 250 x 4 mm, 5 µm particle size). The sample was eluted at 1 mL/min with a linear gradient of 0-66% acetonitrile (ACN) containing 0.1% TFA over 60 min. The solvent delivery and gradient formation over 60 min was achieved using the Shimadzu LC-20AD HPLC system. Eluted fractions were collected manually according to 215 nm absorbance, and dried in a Speed-Vac system. The relative abundances (percentage of the total venom proteins) of the different protein families in the venom were estimated from the relation of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks.

Isolated protein fractions were further assayed by SDS-PAGE on 15% polyacrylamide gels under reducing condition with beta-mercaptoethanol as the reducing agent. The

separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes by using Invitrogen iBlotTM System (Program P2, 8-10 minutes), and the protein bands were made visible by Coomassie Blue R-250 stain. The protein samples were subjected to peptide N-terminal sequencing by Edman degradation method using the Procise Sequencer (Applied Biosystem Sciex) available at the Mission Biotech of Taipei, Taiwan ROC. The N-terminal amino acid sequences determined were subsequently subjected to similarity searches against the available databases using the BLAST search program (www.ncbi.nlm.nih.gov/BLAST), with settings as described for the *de novo* sequencing work (Section 10.2.1).

A separate set of gels from the SDS-PAGE of the reverse-phase HPLC fractions were stained with Coomassie Blue R-250 to determine the molecular masses of purified proteins, and for further in-gel enzymatic digestion for peptide mass fingerprinting as described by Bringans *et al.* (2008). Excised protein bands were destained by 3 cycles of 45-min washes with 25 mM ammonium bicarbonate in 50:50 acetonitrile:water, and the gel plugs/pieces were vacuum-dried and stored at -20 °C. For in-gel protein digestion, 10 µL of digestion buffer (12.5% µg/ml sequencing-grade bovine pancreatic trypsin in 25 mM ammonium bicarbonate) was added to each gel piece and incubated overnight at 37 °C. The tryptic peptides were subsequently extracted by two 20-min incubations with 10-20 µL ACN containing 1% TFA. The extracts were dried by rotary evaporation and stored at -20 °C pending further peptide mass analysis.

Peptide mass analysis was performed by MALDI-TOF/TOF MS/MS on the AB SCIEX TOF/TOFTM 5800 System Proteomics Analyzer (Applied Biosystem Sciex). The peptide samples were reconstituted in 2 µL diluent (30:70 ACN:water). The solution was diluted 1:10 with matrix solution (alpha-cyano-4-hydroxycinnamic acid (Sigma),

10 mg/ml) and spotted on a 384-well Opti-TOF stainless steel plate. The spotted samples were analysed using a first run of standard TOF MS. The system was set to perform a second run of MS/MS focused on the 15 most intensive peaks of the first MS excluding peaks known to be trypsin. The laser was set to fire 400 times per spot in MS mode and 2000 times per spot in MS/MS mode. Laser intensity was 2800 J (MS) and 3900 J (MS/MS). A mass range of 400-4000 u (unified atomic mass unit) with a focus of 2100 u was used. Spectra were analysed to identify protein of interest using Mascot sequence matching software (Matrix Science) with Ludwig NR Database and taxonomy of Serpentes (June 2012, 16,183 sequences). In-gel digestion, tryptic peptides extraction and subsequent peptide mass printings were carried out at Proteomics International, Perth, Australia which provided the mass spectrometry proteomic facility.

10.3 Results

10.3.1 Shotgun-LC-MS/MS and *de novo* sequencing

Figure 10.1 shows that the loaded tryptic peptides were completely eluted at an acetonitrile concentration of 50%. Depending on the population of the constituent peptides, > 1000 MS/MS spectra were automatically collected, and subsequently matched for the most probable proteins or peptides by the software algorithm Mascot. In all cases, the best results are achieved where two or more peptides were mapped to the same protein. A singly-matched peptide at high confidence is nevertheless indicative. The proteins identified via this method were summarized in Table 10.1.

In *de novo* sequencing, sequences with the best *de novo* score (Table 10.2) was submitted for further bioinformatics analysis (BLAST searching) at NCBI BLAST website (*blastp* suite at www.ncbi.nlm.nih.gov/BLAST) using settings suggested for short peptide sequences. When the identity of the top hit for *de novo* peptides was determined, all were matched to known venom proteins listed under the Serpentes or Viperidae taxon in the database. Proteins matched to the first three hits for each *de novo* sequence appeared to be mostly of the same protein family. This indicates that the homology matching of short sequences is likely producing true venom protein matches, above that expected by chance. The identity of protein derived from *de novo* peptide sequencing was further verified based on its putative functional category derived using a simple function-scoring system for each probable protein matched to the top hits (Table 10.2), as modified from Bringans *et al.* (2008) .

By using the shotgun approach including enrichment with *de novo* sequencing method, a total of 52 individual proteins were identified from *H. hypnale* venom. Out of these, 39 were determined by ≥ 2 homology-matched peptides, 4 were determined with single homology-matched peptide, and 9 were identified from *de novo*-derived sequences based on putative functions. Major proteins found in the venom are of secretory types (> 70% by protein identities), which are mainly enzymes with toxinological activities. Three major enzymes commonly present in viperid venoms were recognized: metalloproteases (10), serine proteases (4) and L-amino acid oxidases (4).

The numbers of various types of proteins identified as components of *H. hypnale* venom by the Shotgun approach (including *de novo* sequencing) were illustrated in Figure 10.2.



Figure 10.1 Elution profile of tryptic peptides of *Hypnale hypnale* venom from reverse-phase HPLC monitored at 214 nm.

Table 10.1 Proteins identified from shotgun-LC-MS/MS of trypsin-digested venom proteins.

No.	Accession	Protein	Species	Molecular mass (Da)	Theoretical pI	Peptide sequences matched	Coverage (%)
1	P0DI84	L-amino-acid oxidase	<i>Vipera ammodytes ammodytes</i>	54,989	7.71	YPVKPSEEGK IQFEPPLPPK FWEDDGIHGGK HDDIFAYEKR IQFEPPLPPK DCADIVFNDLSLIHQLPK	12.4
2	P81382	L-amino-acid oxidase	<i>Agkistrodon rhodostoma</i>	58,506	6.05	FWEDDGIHGGK HDDIFAYEKR LNEFSQENDNAWYFIK DCADIVFNDLSLIHQLPK	10.7
3	B5AR80	L-amino-acid oxidase	<i>Bothropoides pauloensis</i>	57,086	6.31	TNCSYMLNK IKFEPPLPPK FWEDDGIHGGK HDDIFAYEKR	8.0
4	A6MFL0	L-amino-acid oxidase	<i>Demansia vestigiata</i>	59,159	7.16	AHGWIDSTIK AHGWIDSTIK DVNRASQKPSR FWEDDGIHGGK	
5	P26324	Serine protease ancrod	<i>Agkistrodon rhodostoma</i>	27,105	8.61	VLCAGDLR VMGWGSINR	17.1

Table 10.1, continued

						RMNLVFGMHR VIGGDECNINEHR	
6	P85109	Thrombin-like enzyme kangshuanmei	<i>Agkistrodon halys brevicaudus</i>	26,967	8.27	FFMYIR WDKDIMLIR WDKDIMLIR WDKDIMLIR VIGGDECNINEHR	11.9
7	P47797	Serine protease ancrod-2	<i>Agkistrodon rhodostoma</i>	29,724	8.64	ILCAGDLQGR WGEDIMLIR WGEDIMLIR	7.4
8	Q7SZE2	Bradykinin-releasing enzyme KR-E-1	<i>Gloydus ussuriensis</i>	25,887	4.82	DIMLIKLSR DIMLIKLSR DIMLIKLSR VIGGDECNINEHR	9.8
9	P0C6E3	Zinc metalloproteinase-disintegrin bilitoxin-1	<i>Agkistrodon bilineatus</i>	33,304	5.28	FKGAGTECR CTGQSADCPR	6.5
10	P30403	Zinc metalloproteinase-disintegrin (rhodostoxin-rhodostomin)	<i>Agkistrodon rhodostoma</i>	55,075	5.6	HSNDLEVIR CTGQSADCPR	4
11	Q9IAY4	Zinc metalloproteinase H4	<i>Agkistrodon acutus</i>	40,767	5.64	VNDYEVVYPR	2.8

Table 10.1, continued

12	Q2LD49	Zinc metalloproteinase-disintegrin TSV-DM	<i>Viridovipera stejneri</i>	70,513	6.4	IACEPENVK	1.4
13	C6G1U7	Neurotrophin-3	<i>Hypsiglena chlorophaea</i>	19,539	8.63	DGVPTTVRK TLIQATIWK TLIQADIWK TLIQADIWK TLIQAMIWK TLIQAFIWK TLIQAFIWK TLIQACIWK TLIQACIWK TLIQACIWK TLIQARIWK	10.5
14	D6P6B6	Neurotrophin 3	<i>Typhlops sp.</i>	24,389	9.74	FAEGKSHR DWIQTTVK TLIQADIWK TLIQADIWK WVTDKSSAVDCR	17.4
15	D6P679	Neurotrophin 3	<i>Typhlops anchaureus</i>	24,513	9.72	AEPDAAAHK FAEGKSHR DDVQTTGKR TLIQADIWK TLIQADIWK	16.4

Table 10.1, continued

16	D3J183	Neurotrophin 3	<i>Mitophis pyrites</i>	18,639	8.78	FAEGKSHR TLIQADIWK TLIQADIWK QQRQFSSPR	15.8
17	Q9PSM4	Galactose-specific lectin	<i>Lachesis stenophrys</i>	16,626	5.05	NQPDHYEGK DFSWEWTDR DFSWEWTDR DFSWEWTDR KDFSWEWTDR KDFSWEWTDR	14.1
18	Q6X5S0	C-type lectin 7	<i>Echis pyramidum leakeyi</i>	17,493	6.51	ADV VWIGLR	6.1
19	P81114	Alboaggregin-A subunit 4	<i>Cryptelytrops albolabris</i>	14,679	4.62	PWREQFECLVSR DCPSDWSSYEGHCYR DCPSDWSSYEGHCYR	22
20	D2YW39	Rhodocetin subunit gamma	<i>Agkistrodon rhodostoma</i>	15,957	5.69	TWDEAER RPELYVWIGLR	13.4
21	Q9DEA2	Agkicetin-C subunit alpha	<i>Agkistrodon acutus</i>	18,109	8.83	TWEDAER IKPYVWIGLR	11

Table 10.1, continued

22	B6EWW5	Aminopeptidase N	<i>Agkistrodon halys brevicaudus</i>	112,390	5.4	ALSNMPIK DNEDVGFGSATR MLSEFLTEAVFR	3.2
23	A5HUI5	Aminopeptidase A	<i>Agkistrodon halys brevicaudus</i>	110,497	5.54	RYTLNDR VNYEPQVWR AVVAVSIGQMVEDDK	3.3
24	F8S101	Phospholipase B	<i>Crotalus adamanteus</i>	64,284	8.66	QDEWTR DDPFWR DDPFWR VTDMESMK NGYWPSYNIPFDK EIYNMSGYGEYVQR	8.5
25	A8QL55	Complement-depleting factor	<i>Ophiophagus hannah</i>	185,524	6.12	VLMAASTGK SDLWPMKDK GALMLKICMR LSEGVDRIYSR IPILDGDGEATLKR DTCMGMLVVKGASSR	4.1
26	A0RZG1	Complement protein C3-1	<i>Austrelaps superbus</i>	185,821	6.17	VLMAASTGR AAQFQDQNLK ASVRGELASDGVR LEGDPGARVGLVAVDK	2.9

Table 10.1, continued

27	D9J014	NADH dehydrogenase subunit 4	<i>Acrochordus granulatus</i>	25,250	9.04	GMQVLPTTK GMQVLPTTK GMQVLPTTK GMQVLPTTK GMQVLPTTK AMQVLPTTK GMQVLPTTK GMQVLPTTK RMQVLPTTK CMQVLPTTK WMQVLPTTK WMQVLPTTK`	
28	Q8HI16	NADH dehydrogenase subunit 4	<i>Contia tenuis</i>	25,482	8.99	LAGYGICR LAGYGIWR LAGYGIWR LAGYGICRMMQTLPTMK	7.4
29	Q8WFF7	NADH-ubiquinone oxidoreductase chain 2	<i>Crotalus atrox</i>	37,315	10.2	NPLWLTVR IITLAKMMK IITLAEMMK	5
30	Q64G16	G protein-binding protein	<i>Oxyuranus scutellatus scutellatus</i>	23,484	9.85	IMVVPSAK YGDSLVR MCTIIKR MCTIIKR KIMVVPSAK	11.7

Table 10.1, continued

31	A9QXJ8	Mitochondrial uncoupling protein B	<i>Rhabdophis tigrinus</i>	33,870	9.81	EEGVRGLWK GLWKGTLPNVS MLQDEGPLAFYK	9.4
32	F2WUN8	ATP synthase subunit a	<i>Crotalus sp.</i>	24,979	9	MFLSMTSQLTPK LLGNRMTTAITGLLK	12.4
33	F8UIL6	A kinase anchor protein 9	<i>Trachyboa boulengeri</i>	53,899	5.04`	VKLDLEENK TELLEKQMK DAVLDRMAESQEAELER	7.6
34	P0CV92	Glutaminyl-peptide cyclotransferase	<i>Crotalus atrox</i>	7,894	6.44	LIFFDGEEAFVR	16.9
35	B4YHB7	Recombination activating protein 1	<i>Vermicella intermedia</i>	41,137	9.03	KGMLNSQLSK KGMLNSQLSK KGMLNSQLSK VMLNSQLSKK TMLNSQLSKK	3.1
36	Q5FY47	Calbindin D28K	<i>Pantherophis guttatus</i>	30,275	4.72	FQGIKMCCK KNIMALSDGGK LELTEMARLLPVQENFLLK	14.9
37	D0VY30	Eukaryotic translation elongation factor 2	<i>Leiolepis reevesii rubritaeniata</i>	36,276	8.61	KVEDMMK DKEGKPLLK EGKPLLKAVMR IFSGVVSTGLKCR IFSGVVSTGLKCR	10.2

Table 10.1, continued

38	D2IL03	Dynein axonemal heavy chain 3	<i>Leptodeira annulata rhombifera</i>	28,442	7.7	VAEVVAPK LISGLGGEK VAAVVAPKR VASVVAPKR VASVVAPKR	7.3
39	D2IL04	Dynein axonemal heavy chain 3	<i>Leptodeira bakeri</i>	28,476	7.7	KFMGDLK LISGLGGEK	6.5
40	F8V4K2	Prolactin receptor	<i>Lampropeltis pyromelana</i>	7,827	8.15	HLSETLK CLSETLK DPEEVCR DPEEVTRNTDR	26.5
41	B8QC49	Aryl hydrocarbon receptor	<i>Liotyphlops albirostri</i>	17,269	4.17	SNKIVLER SNKINLER SNKIHLE	5.4
42	B7TXS4	RAG2	<i>Duberria lutrix</i>	26,298	5.09	MIVIIGGR MIVIIGGRSYMALR	6
43	D0VXZ7	SRY-box 9	<i>Leiolepis reevesii rubritaeniata</i>	22,573	9.95	LVPMPVR LVPMPVR RPFVEEAERLR	9

Table 10.2 *De novo* peptide sequences and their protein functional categories.

No. of sequence	Precursor ion mass (Da)	<i>De novo</i> Sequence	<i>De novo</i> score (%)	Protein functional category
1	1344.57	FQSVGVVQDHTK	100	Snake venom metalloproteases - III
2	1597.56	TGALVVQNPAECCHR	100)	R35 (cellular protein)
3	1202.49	FDDVLDPEPR	100	Snake venom serine proteases
4	1136.55	YNDDLEVLR	100	Snake venom metalloproteases - I
5	1200.59	FDDVLDPEPR	100	Snake venom serine proteases
6	1653.76	HEMGHNLGLHHDEK*	96	Snake venom metalloproteases - III
7	1526.74	GGFNPDYEVLYPR	95	Snake venom metalloproteases - II
8	1098.41	LVCCGDDMDK	62	Disintegrins
9	2898.49	VFSSDHAAVNVLVADESMVKKPGAVPCK	61	Snake venom metalloproteases - I
10	2118.08	NSEDPATVTLVLVADEFLGK	50	Snake venom metalloproteases - III

* with putative conserved domains of ZnMc Superfamily

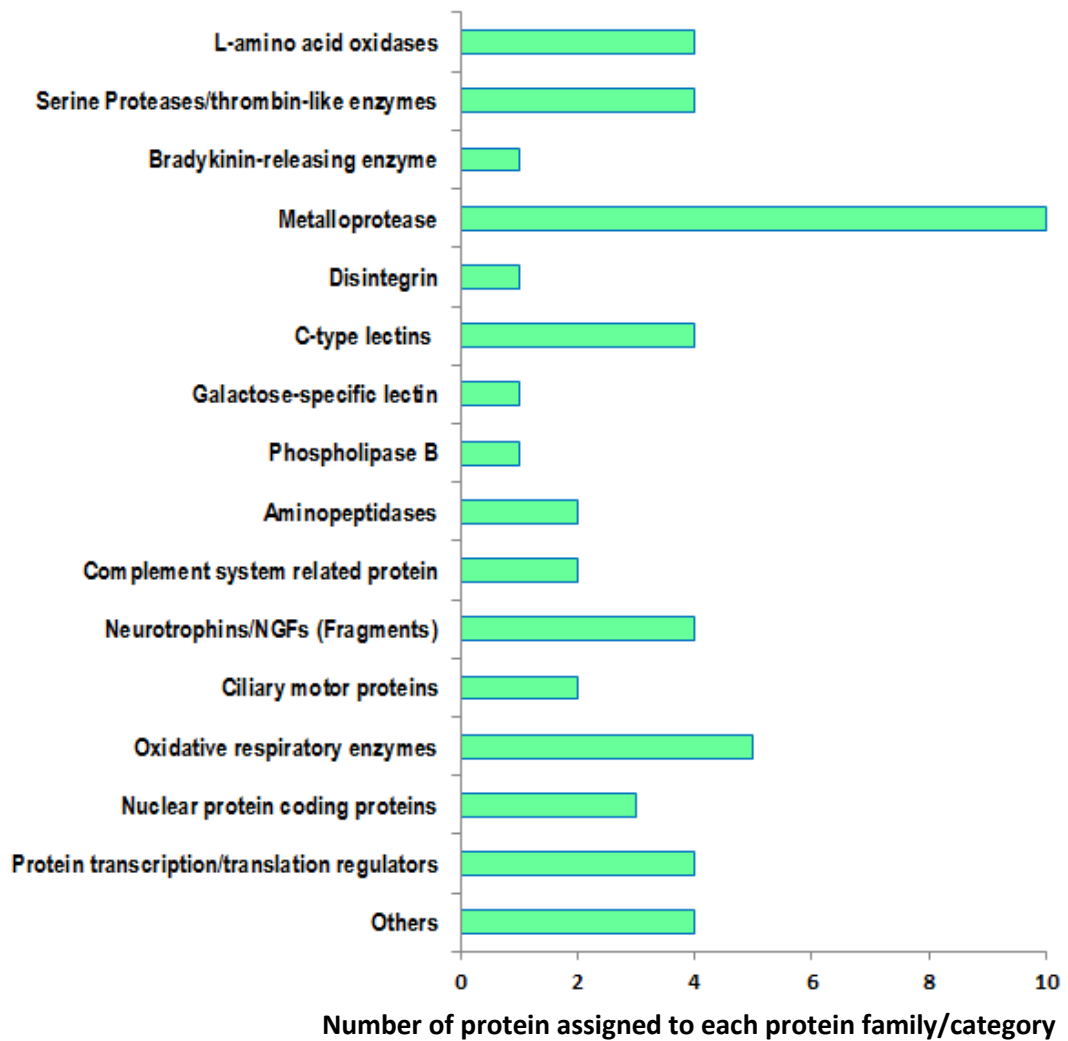


Figure 10.2 Number of various proteins identified by shotgun-LC-MS/MS approach coupled with *de novo* peptide sequencing. 70% of the proteins represent the putative toxins in the venom responsible for most biological effects including envenoming pathology. The figure illustrates the complexity expected for the venom, without indicating the abundance or composition percentage of each protein.

10.3.2 Sequential use of reverse-phase HPLC, SDS-PAGE and peptide sequencing

10.3.2.1 Reverse-phase HPLC separation of venom proteins and SDS-PAGE

Reverse-phase HPLC resolved the venom into 12 major peaks as monitored at 215 nm absorbance. Proteins were present in 10 peaks as determined by Bradford method and SDS-PAGE under reducing condition (Figure 10.3).

10.3.2.2 Peptide sequencing for protein identification

There were approximately 20-24 distinct proteins identified through the N-terminal and/or internal sequences. The proteins nevertheless are limited to only several families expected from snake venoms. Among these are the common ones like the zinc-dependent metalloproteases, serine proteases/thrombin-like enzymes, phospholipases A₂, L-amino acid oxidases, C-type lectins.; and the less commonly reported nerve growth factors. Besides, aminopeptidases and low-molecular mass proteins with sequences similar to those of three-finger toxins were also identified, but these two protein groups were present in relatively low abundance (< 2%).

The assignment of the various proteins identified was tabulated in Table 10.3. Figure 10.4 illustrates the composition of the major protein families identified based on their percentage of occurrence in the venom.

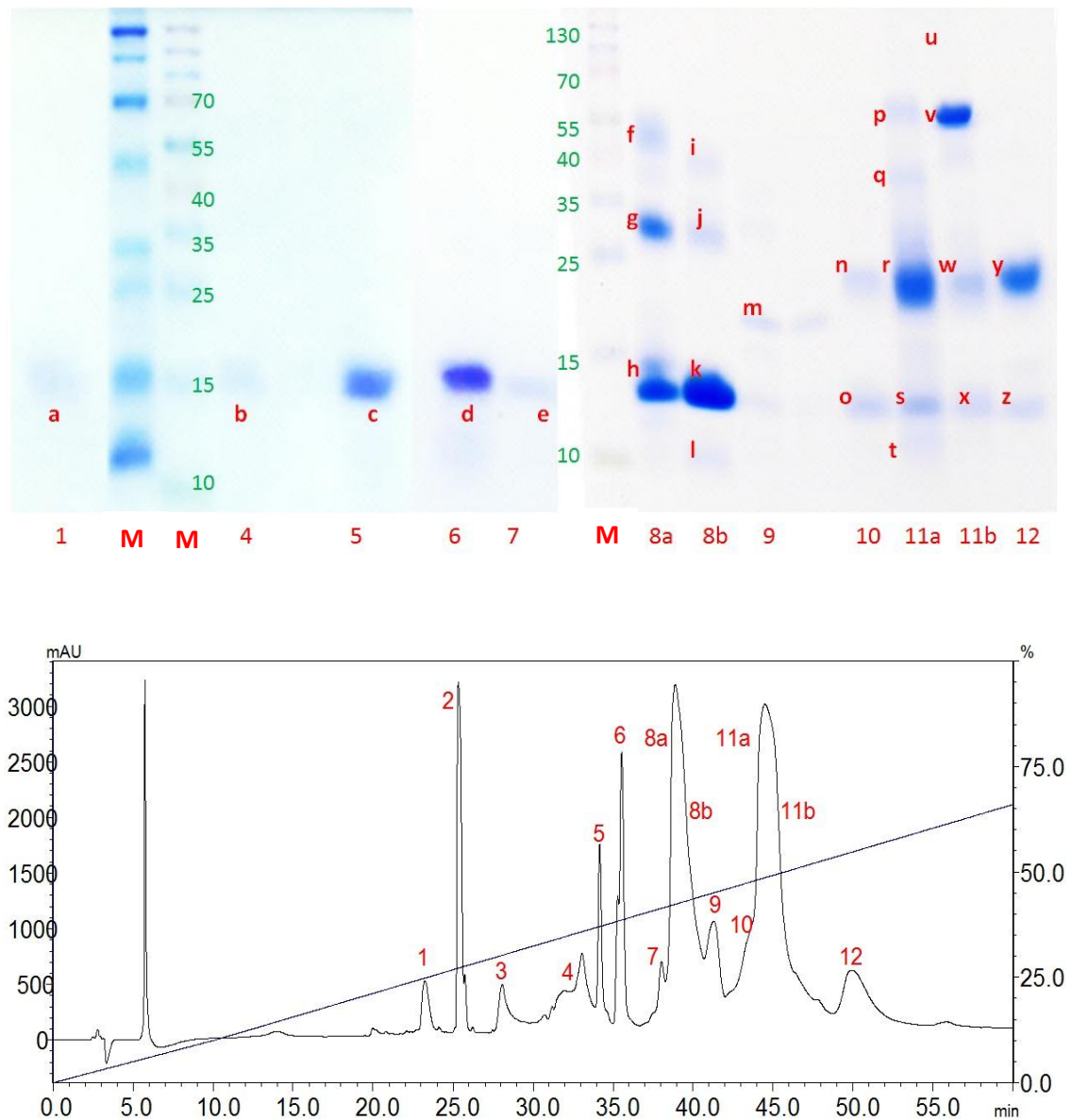


Figure 10.3 Reverse-phase HPLC separation of *Hypnale hypnale* venom and further separation by 15% SDS-PAGE under reducing condition. Chromatogram in the lower panel shows the venom fractionation by reverse-phase HPLC (absorbance: 215 nm) that yielded 12 major peaks of which 10 were peptidic with significant amount of proteins (fractions 2 and 3: non-peptidic). The proteins were further separated by reducing SDS-PAGE as shown in the upper panel. Peaks (fractions) were numbered 1-12 (8a and 11a were collected from the ascending slope; 8b and 11b from the descending slope). On the SDS-PAGE profile, each visible protein band was alphabetically labelled. Protein molecular markers (M) were in kDa molecular masses.

Table 10.3 Assignment of the reverse-phase HPLC and SDS-PAGE fractions of *Hypanel hypnale* venom to major protein families by N-terminal Edman sequencing and/or peptide mass fingerprinting (MALDI-TOF/TOF mass spectrometry) of selected peptide ions from in-gel digested protein bands separated by SDS-PAGE as shown in Figure 10.3.

Fraction	Band	N-terminal sequence	MALDI-TOF/TOF sequence	Protein family	Molecular mass (kDa)
1	a	EKEE	No matches	unknown	15
2 & 3	-	-	-	(non-peptidic)	-
4	b	--	No matches	unknown	15
5	c	HLLQFESMIK	--	Phospholipase A ₂	15
6	d	HLMQFELMIMHET GK	--	Phospholipase A ₂	15
7	e	HLLQFESLMIM	FAELLTKFSYR*	Phospholipase A ₂	15
8a	f	IIGGDP	--	Serine protease (thrombin-like enzyme)	48
	g	--	LQGETYFIEPMK NNDEPPQK*	Zinc-dependent metalloproteases PII (homology with rhodostoxin)	31
	h	NLWDLWKMVM	--	Phospholipase A ₂	14
8b	i	VIGGDECNIN	VMGWGSINR EKYFFR	Thrombin-like enzyme ancrod (serine protease)	37
	j	--	--	(Deduced as zinc-dependent metalloprotease PII)	31
	k	NLWDLWKMVM	SDPNPDTTK* MVSAMTNR*	Phospholipase A ₂	14
	l	--	DGENLCVKR IFLTCTTKFWEDDGIHGGK*	Low molecular weight protein (matched to 3-finger toxin)	8
9	m	--	RQFQSPR TEAGLELTTSQVTK	Venom nerve growth factor/neurotrophin	21
10	n	--	RSWTAVR IENHEGVR*	Zinc-dependent metalloproteases (likely PII)	24
	o	--	FEPQGECSAW	C-type lectin	14

Table 10.3, continued.

11a	p	ADD-	K.SAGQLYEESLGK.V K.FNPPLLPK.K	L-amino acid oxidase	60
	q	--	SVGIVR R.SVGIVR.D R.VALIGTEIWSTGNLSK.V	Zinc-dependent metalloproteases	40
	r	TLELLGD	R.SFGEWR.E K.VPDSEAHAVYK.Y K.GEHDPMERPASAK.G K.GEHDPMERPATAK.G	Zinc-dependent metalloproteases (likely PII)	25
	s	--	K.RPELYVWIGLR.D	C-type lectin (rhodocetin)	14
	t	--	GCAATCPKMK IFLTCTTKFWEDDGIHGGK	Low molecular weight protein (matched to 3-finger toxin)	9
11b	u	--	AMEVYDRVADVAPK SAVYCSAIR	Aminopeptidase	95
	v	--	K.HDDIFAYEKR.F K.KFWEDDGIHGGK.S R.IYFAGEYTAQAHGWIDSTIK.S	L-amino acid oxidase	60
	w	--	K.KESLQDALFR.R R.VTLVGLEFWSNR.D K.FIKLVIVVVHSMVTK.Y	Zinc-dependent metalloproteases (likely PII)	25
	x	--	K.DFSWEWTDR.S K.KDFSWEWTDR.S	C-type lectin	14
12	Y	SLLLQA*	KWSDQNCR NELQRNGQPCLK	Zinc-dependent metalloproteases (likely PII)	25
	z	--	K.NAFLCQCKF K.DFSWEWTDR.S K.KDFSWEWTDR.S	C-type lectin	14

-- peptide sequences not determined due to terminus blockage and/or limitation of protein amount

** peptides with <100% sequence coverage

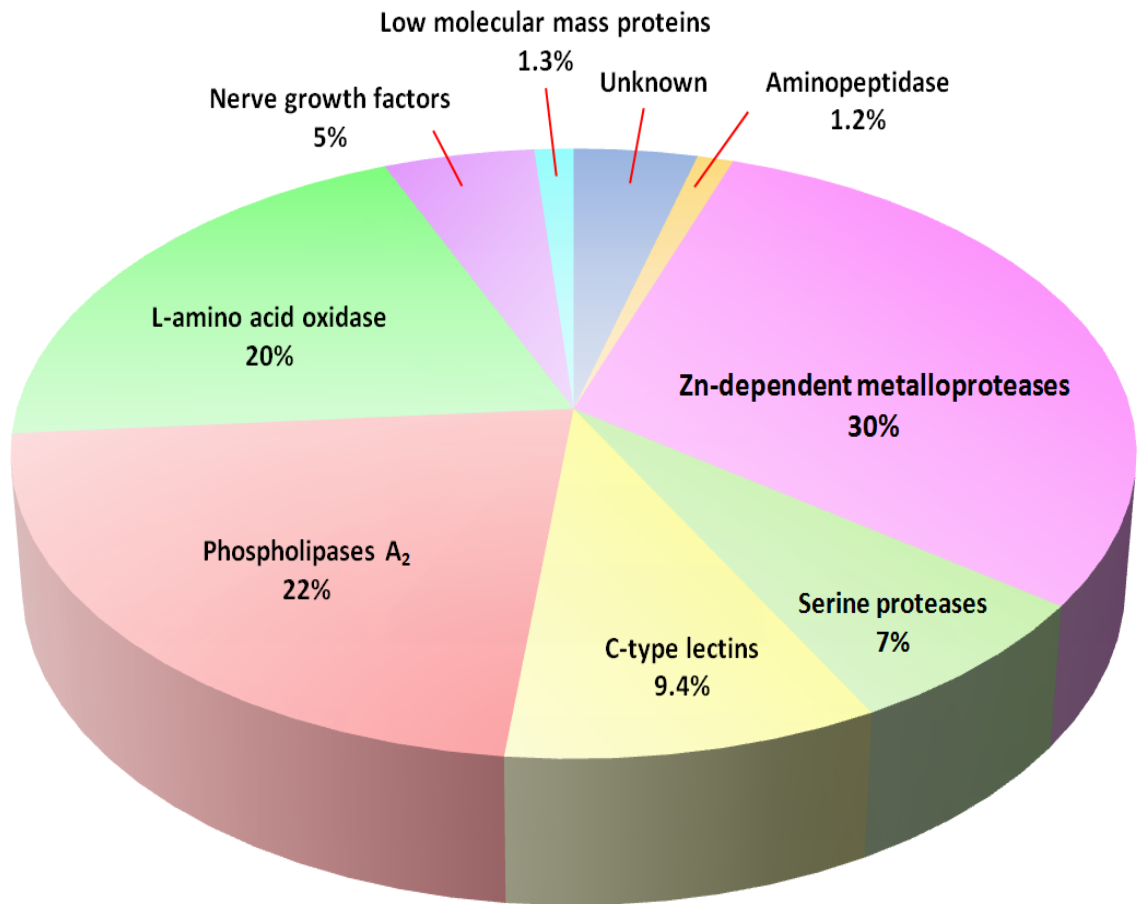


Figure 10.4 Overall protein compositions (relative abundance in %) of *Hypnale hypnale* venom according to major protein families. Venom separation was achieved by reverse-phase HPLC separation of the venom followed by SDS-PAGE of the fractions; and protein categories were identified through peptide mass fingerprinting and/or N-terminal sequences.

10.4 Discussion

10.4.1 Shotgun-LC-MS/MS approach in *Hypnale hypnale* venom study

In this approach, direct protein digestion with trypsin was carried out, prior to separation of the resultant tryptic peptides by liquid chromatography. This method was thought to be able to minimize the loss of proteins and peptides as compared to multiple-step protein separation prior to digestion. Shotgun-LC-MS/MS method reported by Li *et al.* (2004) indeed identified ~80 proteins from *Naja atra* venom and 28 proteins from *Agkistrodon halys* venom. Fry *et al.* (2003c) identified 2 to 26 proteins from various undigested snake venoms by using an LC-MS/MS approach. From a spider venom, dos Santos *et al.* (2009) recovered 36 proteins with a similar shotgun approach. This approach provides information on the types of proteins from a mixture of protein (venom). However, the shotgun-LC-MS/MS method does not determine the relative abundance of the different protein components, hence limiting the correlation of major toxin components with the pathophysiology of envenoming.

In addition, it is known that results generated by automatic database searching were not absolute, especially when considering the fact of unavailability of species-specific genomic sequences. In situations where no significant hits were obtained, it is possible that the amount of certain peptides was insufficient, or there is lack of homologous sequences in the database (Bringans *et al.*, 2008). *De novo* sequencing hence provides an alternative approach, whereby internal sequences of peptides were generated based on the 'best fit' to the MS/MS data after considering the complexity of the spectra and numerous possible a, b, c, x, y, z ion combinations. Conventional BLAST searching assigns an E-value (Expect value for a 'chance occurrence') to any possible sequence mapping (between the query peptide and database), and generally a lower E-value

predict a better homology match. However, the true meaning and usefulness of E-value is controversial when a short *de novo* sequence is matched against a large database where with a lack of species-specific genomic information. In this *de novo* sequencing-BLAST approach, the stringency of search parameter was therefore lowered by adopting a higher E-value threshold that allows reporting of low scoring matches.

As indicated, most of the proteins identified could be categorized into a few major types with conserved molecular structures respectively. The finding supported the view that venom proteins belong to only several major protein families as a consequence of the recruitment of a restricted set of proteins into the venom proteome before the diversification of the advanced snakes at the base of the radiation of Colubroidea (Vidal, 2002; Fry and Wüster, 2004; Calvete *et al.*, 2009). By comparison with other snake venom proteomes, the occurrence of multiple isoforms with function variations within each major protein family evidences the emergence of paralogous groups of genes (for gene duplications that led to molecular novelties) before the divergence of the various snakes (Calvete *et al.*, 2009). The predominance of certain protein families nevertheless can still be anticipated in some phylogenetically related snake venoms.

Majority of proteins identified from *H. hypnale* venom using the shotgun-LC-MS/MS and *de novo* sequencing approach belong to the enzyme families of metalloproteases, serine proteases and L-amino acid oxidases. Like many viperid venoms, *H. hypnale* venom contains diverse isoforms of snake venom metalloproteases (SVMPs). All 10 metalloproteases identified belong to the zinc-dependent snake venom metalloprotease family, with a breakdown by subtypes as SVMP-I (3), SVMP-II (3) and SVMP-III (4). Generally, SVMP-I is proteolytic (tissue-damaging) without significant hemorrhagic effect, while SVMP-II (with disintegrin domain) and SVMP-III (with disintegrin and

cysteine-rich domain), cause extensive hemorrhages over the bitten site in addition to systemic bleeding tendency, hence the toxin name of ‘hemorrhagin’ in some literatures. The hemorrhagic action is mainly due to proteolytic damages on connective tissues, basal membranes and vasculature, as well as anti-platelet activities of the SVMPs (Fox and Serrano, 2008). Several snake venom serine proteases (SVSPs) too were identified in the venom. Not surprisingly, serine protease peptides of *H. hypnale* venom were homologously mapped to thrombin-like enzymes (ancrod-1 and ancrod-2) from the Malayan pit viper, *Calloselasma rhodostoma*, a close sister taxon of the former (Parkinson *et al.*, 1997). Similar venom antigenic properties between the venoms of *H. hypnale* and *C. rhodostoma* have been indicated and supported by cross-neutralization as well as cross-reactivity studies discussed in Chapters 5, 6 and 8. It is noted that 4 SVSPs identified were functionally related to thrombin-like/procoagulant activity, mediated through fibrinolytic action that frequently complicates hypofibrinogenemia, leading to venom-induced consumptive coagulopathy. In addition to these 4 thrombin-like SVSPs, one protein shows homology with a bradykinin-releasing SVSP, indicating this possible biological function of the venom which has not been characterized previously. Several isoforms of L-amino acid oxidase were also identified. This class of enzyme possesses diverse biological properties which include cytotoxic and potentially hemorrhagic activities (Tan and Fung, 2010) that contribute to the envenoming pathophysiology.

Disintegrins and lectins (galactose-specific, C-type lectins) are less abundant components commonly present in hemorrhagic venoms especially from the Viperidae snakes. Their pharmacological actions are mainly directed on platelet activities, either of potentiating or inhibitory effect. Alboaggregatin (Peng *et al.*, 1991) and rhodocetin (Wang *et al.*, 1999a), two C-type lectins isolated from Asiatic pit viper venoms

(*Cryptelyptrops albolabris* and *C. rhodostoma*, respectively), were among those with homologies mapped to *H. hypnale* venom peptides. The effect of a platelet aggregation-inhibiting toxin is self-explanatory, but for a platelet aggregation-inducer, the ultimate systemic outcome is usually still related to a hemorrhagic syndrome as aggregation of functional platelets leads to thrombocytopenia. This is a feature frequently reported in pit viper envenomings, including that for *C. rhodostoma* and *H. hypnale* bites (Reid *et al.*, 1963; de Silva *et al.*, 1994; Joseph *et al.*, 2007).

The shotgun-LC-MS/MS approach also identified aminopeptidases, very high molecular mass enzymes with presumably peptidolytic/proteolytic function that aids in extracellular matrix digestion of prey and, potentially contributes to tissue-damaging effect in envenoming cases. It too identified complement system-related proteins, which are possibly involved in inflammatory responses and immunological reactions. The approach however, failed to detect the presence of phospholipase A₂ which is commonly present in snake venoms, although the system identified the presence of one phospholipase B. A possible explanation for this is ion suppressions by other peptides of similar masses (Fry *et al.*, 2003(c)); further optimization of the instrumentation and repetition of the study were possible but unfortunately would incur very high cost. Instead, we proceeded to explore the proteome using another multi-dimensional approach (see Section 10.4.2 discussion on the combined use of HPLC, SDS-PAGE, peptide sequencing & MS), which successfully determine the composition of phospholipases A₂. In fact, the enzyme activity has been readily identified via functional (biochemical) study as reported in Chapter 4, suggesting that multiple approaches and triangulation of data are essential in studying the venom composition and functions. Other proteins and enzymes not identified at this stage include phosphodiesterase, phosphomonoesterase, hyaluronidase, nucleotidase, and protease inhibitors etc., which

are expected to be low in abundances and have limited species-specific information on the database. Nevertheless, many of these proteins, like PLA₂ enzyme, have been screened for their activities on various enzymatic assays as described in Chapter 4. The absence of some of these proteins in proteomics reports reflects the limitation of venom proteomic studies that rely heavily on the quality of mass spectrometry results and the completeness of a snake gene/protein database while in reality not all sequence information are readily available.

Nerve growth factor (NGF), a member of the neurotrophin family was identified in venom *H. hypnale* venom through the current shotgun-LC-MS/MS analysis. The presence of NGFs in snake venom seems somewhat incongruous, as these are stimulatory proteins for nerve growth that obviously contradict the killing and digestive functions of a venom (Cohen and Levi-Montalcini, 1954). It however remains possible that snake venom NGFs at predatory doses may exhibit neurotoxic effects on selected preys. Their presence in many snake venoms has been reported, and neurotrophic activities have been characterized mainly on neuron cell cultures (Lavin *et al.*, 2010). This is indeed another group of snake venom constituents, after the widely-studied anticoagulant, anticancer and antimicrobial toxins, shown to have therapeutic potential related to neurodegenerative or brain disorders, e.g. Alzheimer's disease (Blesch and Tuszynski, 2004; Koh *et al.*, 2006).

Using the shotgun-LC-MS/MS method, a number of proteins mapped to tryptic peptides of *H. hypnale* venom were found to be of cellular types, with functions mainly involved in energy production, protein synthesis and ciliary movement. Functions of the majority of these proteins are relevant to the physiology of snake venom gland, a specialized organ comprised of secretory cells, myoepithelial cells and mitochondria-rich cells, with

numerous of these being ciliated (Weinstein *et al.*, 2010). The glandular cells are highly active in synthesis of venom proteins (enzymes and toxins) which are stored in the glandular lumens and delivered (expelled) via ducts and fangs into recipient tissues during envenomation. The presence of these cellular proteins in the venom might be due to an incidental release of these proteins from the cells, possibly through ruptured or dislodged cells induced by the milking process. Nevertheless, the amount of these proteins was believed to be small; the fact that the protein hits were significant from the automatic database search could be due to a more established databanks of cellular physiological proteins where higher degrees of homology is expected to be conserved across various taxonomic lineages, in contrast to venom proteins that have undergone rapid sequence amino acid divergence under accelerated evolution (Deshimaru *et al.*, 1996; Ohno *et al.*, 1998; Ogawa *et al.*, 2005).

The wide variety of major enzymes e.g. metalloproteases, serine proteases/thrombin-like enzymes and L-amino acid oxidases present in *H. hypnale* venom correlates well with *H. hypnale* envenoming syndrome and the venom toxinological profiles. Procoagulant enzymes, SVMP-II, SVMP-III, lectins as well as disintegrins, were often conveniently categorized as “hematoxic components” of venom to highlight (or warn against) their pathological effects on platelet activities and blood clotting mechanisms, particularly in clinical setting. Systemic envenoming by *H. hypnale* is generally characterized by hemostatic disturbances and infrequently, acute kidney injury, a life-threatening complication likely secondary to consumptive coagulopathy. In addition, the presence of cytotoxic and cytolytic enzymes including SVMPs and LAOs correlates with the severe local tissue inflammation and necrosis (Viperine syndrome of local envenomation), features consistent in the majority of *H. hypnale* envenoming cases (Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008).

10.4.2 Sequential use of reverse-phase HPLC, SDS-PAGE and peptide sequencing for protein identification and abundance estimation

In contrast to the shotgun-LC-MS/MS approach, sequential venom separation by reverse-phase chromatography and SDS-PAGE prior to mass spectrometry analysis has allowed the estimation of the relative abundances (percentage of the total venom proteins) of different protein families present in *H. hypnale* venom. Knowledge of the constituents and their compositions in snake venom is potentially useful for a better understanding of the envenoming pathophysiology, treatment (antivenom) design, and novel drug discovery.

MALDI-TOF/TOF mass spectrometry was used for peptide mass fingerprinting with automatic database search. This approach unfortunately was not able to assign all the tryptic peptides (in-gel digests of each electrophoretic band) to known protein families due to their low significance of protein score. The following proteins were affected: band g (peak 8a), bands k and l (peak 8b), band m (peak 9), band n (peak 10), band t (peak 11a) and band y (peak 12). Despite the low significance, there were generally at least 3-5 peptides which sequences were determined with protein hit ranked number 1. These represented sequences present within a sample, however unfortunately did not match existing proteins in the database. This is expected from the rapid amino acid sequence divergence of venom proteins, even within a same protein family, and reflects molecular adaptation of venom proteins under accelerated evolution (Deshimaru *et al.*, 1996; Ohno *et al.*, 1998; Ogawa *et al.*, 2005). Hence, it is rational to revise manually these MALDI-TOF/TOF peptide ion sequences by submitting them to BLAST similarity searches. The manual approach (with BLAST analysis) has been adopted in similar situations where MALDI-TOF peptide mass fingerprinting could not identify

any protein in the existing database of snake venom proteins (Angulo *et al.*, 2008; Calvete *et al.*, 2007a). Based on the approach, following BLAST similarity searches, the most probable proteins for peptides with low significance scores in the current study were determined based on the peptide sequence coverage and molecular mass.

In this study, zinc-dependent metalloproteases appear to be the most abundant constituent (30%) in *H. hypnale* venom. Snake venom metalloproteases (SVMPs) are known for diverse actions resulting in local and systemic envenomations. Clinical features such as inflammation (swelling and pain), wound hemorrhage, blistering, and irreversible tissue destruction i.e. necrosis are not uncommon. The pathophysiological actions have been well characterized: disruption of capillary vessel integrity, matrix degradation, dermal-epidermal junction separating, secretion of proinflammatory mediators; all lead to tissue ischemia, disrupted tissue repair, and a varied degree of tissue damages (e.g. dermonecrosis, myonecrosis) (Gutiérrez *et al.*, 2010). Indeed, the local effects can be synergistically ‘enhanced’ by the presence of cytolytic toxins e.g. L-amino acid oxidases and phospholipases, which in *H. hypnale* venom represent the other two most abundant components ($\geq 20\%$ of total composition, respectively). High abundance of these moderate to high molecular weight proteins (SVMPs, PLA₂s and LAAOs) which are able to exert their actions primarily on local tissues (surrounding a bite site) correlates with the severe tissue damages reported (de Silva *et al.*, 1994; Ariaratnam *et al.* 2008). The intensive interaction of most venom components with the local tissue accompanied by consequent tissue destructions may also explain for the low systemic absorption of the venom injected intramuscularly in rabbits (Section 9.4.1).

When absorbed systemically, the SVMPs (specifically the SVMP-II and SVMP-III subtypes) can induce systemic effect associated with hemorrhagic syndrome: mediated

by their proteolytic actions that result in microvasculature damages, activation of Factor X and prothrombin, inhibition of platelet functions and von Willebrand factor (Markland, 1998a; Gutiérrez *et al.*, 2010). The potent tissue-damaging property of SVMPs also suggests its possible involvement in end-organ damage, e.g. acute kidney injury. The renal complication could be resulted from SVMPs-induced capillary damages that lead to impaired renal hemodynamics, in addition to coagulopathy induced by other hematoxic toxins e.g. thrombin-like enzyme. This forms a hypothesis for potential research work in the future to elucidate the complex nephrotoxic activity of the venom.

By analyzing the SDS-PAGE profile and N-terminal sequences, two major forms of snake venom serine proteases (SVSPs) were identified in the venom, which constitute ~7% of the total venom weight. The abundance was comparable to that of ancrod, a major thrombin-like enzyme in *Calloselasma rhodostoma* venom (7%) (Esnouf and Tunnah, 1967), although ancrod is known for a much potent procoagulant activity (Section 4.3.4). SVSPs are important coagulant enzymes that induce defibrinogenation leading to consumptive coagulopathy. SVSPs, hematoxic SVMPs, along with C-type lectins and disintegrins that disrupt normal platelet function (as discussed in Section 10.4.1), are likely responsible for the major systemic envenoming syndrome in *H. hypnale* bites, i.e. blood incoagulability (de Silva *et al.*, 1994; Premawardena *et al.*, 1996, 1998; Joseph *et al.*, 2007; Ariaratnam *et al.*, 2008). The major components of SVSPs and SVMPs from *H. hypnale* venom are however believed to share common antigenic epitopes with those from *C. rhodoselasma* venom, based on the cross-neutralization and immunological studies reported in Chapters 5, 6 and 8.

L-amino acid oxidases usually constitute only 1 to 4% of most snake venoms (Tan, 1998), while venoms from mambas and sea snakes contain either no or only trace amount of LAAOs. In colubrid venoms, LAAO activity is typically absent (Mackessy, 2002). This class of enzyme however exists in a much higher abundance (20%) in *H. hypnale* venom, comparable to that observed in the case of *C. rhodostoma* (up to 30% by weight of the dried venom) (Tan, 1998). The enzyme's primary function in most snake venoms is believed to be of antimicrobial purpose, however, the huge amount of LAAOs in *H. hypnale* and *C. rhodostoma* venoms seem to suggest a more diverse pharmacological and toxinological property of the LAAOs besides antimicrobial activities. Indeed, hemorrhagic, platelet-disrupting, anticoagulant, and apoptotic activities have been reported in several snake LAAOs (Tan and Fung, 2010). Further characterization of LAAOs from *H. hypnale* venom, a rich source of the enzyme, is therefore useful for drug discovery for various therapeutic applications.

In this study with the use of reverse-phase HPLC and SDS-PAGE, phospholipase A₂ was clearly shown to constitute a large proportion of the venom content (22%). Several (~4) isoforms exist, and the N-terminal sequences were consistent to a previous report (Wang *et al.*, 1999b), where 2 subtypes of PLA₂ were recognized based on their residues 6: Glu 6 (E6-PLA₂), and Trp 6 (W6-PLA₂). Wang *et al.* (1999b) reported that E6-PLA₂s inhibited platelet aggregation induced by ADP and agonists, whereas W6-PLA₂s possessed a potential heparin-binding property and edema-inducing effect. The authors also reported the occurrence of the two PLA₂s subtypes in *C. rhodostoma* venom with their distinct activities as described for the PLA₂s of *H. hypnale* venom, and suggested that the findings supported the phylogenetic relatedness of the two Asiatic pit vipers. The W6-PLA₂s indeed shared characteristics of K49-PLA₂ subtypes isolated from many crotalid venoms (presence of L5, W6, Q11, N28, P31, mutations at

G32/G33) including those of New World species with highly conserved primary structures. Although being relatively less active enzymatically, these enzymes are known for membrane-damaging and myotoxic properties (Selistre de Araujo *et al.*, 1996; Ward *et al.*, 1998; Arni *et al.*, 1999; Angulo *et al.*, 2002; Lomonte *et al.*, 2003). Generalized myotoxicity has never been reported clinically in *H. hypnale* envenomations, however, the cytolytic activity of snake venom PLA₂s causing myonecrosis has been widely reported (Gopalakrishnakone *et al.*, 1997; Lomonte *et al.*, 2003). Therefore, in *H. hypnale* envenomations, the E6-PLA₂ and W6-PLA₂ may respectively contribute to haemorrhage (platelet dysfunction) and local myonecrosis, in addition to pathological effects induced by other components in the venom.

On reverse-phase HPLC, the C-type lectins (~15 kDa) were eluted at a later course than the PLA₂s which was unfortunately missed out by the shotgun-LC-MS/MS method possibly due to ion suppression. The eluted lectins included one that is homologous with rhodocetin derived from *C. rhodostoma* venom. The effects of snake venom C-type lectins were mainly on platelet activity impairment as discussed in Section 10.4.1, contributing to hemorrhagic syndrome on the whole. Nerve growth factors and aminopeptidases were also identified, consistent with the shotgun-LC-MS/MS findings but in rather low abundances. Nevertheless, the presence of low molecular mass proteins (~8-9 kDa) shown on the SDS-PAGE profiles for peaks 8 and 11 was intriguing. The identities would have been deduced to be most likely disintegrins, however, MS/MS tryptic peptides of the digests revealed sequences partially matched to 3-finger toxins instead. The occurrence of 3-finger toxins (genes and/or proteins) was recently reported in several viperid venoms of *Atropoides nummifer* (Angulo *et al.*, 2008), *Daboia russelii* (Shelke *et al.*, 2002), *Lachesis muta* (Junqueira-de-Azevedo *et al.*, 2006), and *Sistrurus catenatus edwardsii* (Doley *et al.*, 2008), indicating that the

toxin family may be more widely distributed than previously thought confined to only the elapids and some colubrids (Fry *et al.*, 2003b; Pawlak *et al.*, 2006), hence supporting the view that venom toxins likely evolved from proteins with normal physiological functions recruited into the venom proteome before the diversification of the advanced snakes at the base of the Colubroidea radiation (Vidal, 2002; Fry *et al.*, 2006; Calvete *et al.*, 2007a). The presence of the low molecular mass proteins with possible 3-finger toxins-like structures in *H. hypnale* venom, however, requires further investigations.

The two approaches used in the proteomic study for *H. hypnale* venom appear to complement one another in the identification of important protein contents. A major limitation was on the inability of the currently employed technology to detect some proteins of low abundance which were anticipated, e.g. venom endothelial growth factors, cysteine-rich secretory proteins, vasoactive peptides, kunitz-type inhibitors, enzymes such as hyaluronidases, phosphodiesterases and nucleases, possibly due to an incomplete specific venom protein database. Further investigations are therefore required to study in-depth proteome and subproteome of the venom, with combined tools for a more sophisticated multidimensional fractionations (e.g. gel filtration chromatography, followed by 2D-electrophoresis and subsequent LC-MS/MS). The results nevertheless provide valuable insights into the composition of toxins correlated with the envenoming syndrome, and highlight the following concerns:

- (1) The toxin compositions were comparable to those of many Asiatic vipers and pit vipers. Particularly noteworthy are some homologous peptide sequences conserved among venoms of *H. hypnale* and *C. rhodostoma*;

- (2) Substantial amounts of SVMPs, SVSPs, C-type lectins and E6-PLA₂s are related to hematoxic complications;
- (3) SVMPs, W6-PLA₂s and LAAOs are likely responsible for cytotoxicity, hemorrhage and necrosis leading to local tissue destruction;
- (4) Antivenom designed/proposed should aim to neutralize the prominent toxins including their subtypes, for instance, both the E6- and W6-PLA₂s must be targeted.
- (5) Unusually large amount of LAAOs suggests potentially diverse pharmacological properties which may worth further characterizations.

CHAPTER 11

Isolation and Characterization of Hypnobin, a Thrombin-like Enzyme

11.1 Introduction

Repeated gene duplication over evolutionary time gave rise to a structurally conserved protein family within which a diverse set of pharmacologies present. It is by such mechanism that the multigene family of snake venom serine proteases (SVSPs) is accounted for (Deshimaru *et al.*, 1999). Numerous SVSP subsets exist, and one of these is functionally related to thrombin and conventionally called thrombin-like enzymes (TLEs). Substantial amount of TLEs are commonly found in venoms of viperid and crotalid snakes including *Calloselasma rhodostoma* and *Hypnale hypnale*. Despite the high degree of inter-species TLE mutual sequence identity (> 60%), there are phylogenetical and geo-trophic factors that lead to variations in their functional “thrombin-like” properties (Kini, 2005; Mackessy, 2010b). Biomedical studies of individual TLE is therefore important for defining the structure-function relationship of its coagulant action, therefore advancing the knowledge on envenoming pathology, treatment improvement, and potential drug discovery.

As shown in Chapter 5, the procoagulant activity of *H. hypnale* venom, in addition to lethality, was effectively neutralized by antivenoms (monovalent and polyvalent) raised against *Calloselasma rhodostoma* venom. This indicated the presence of similar antigenic properties between venom TLEs of the two sister taxa. While the TLE isolated from *C. rhodostoma* venom has been studied for almost half a century (Esnouf and Tunnah, 1967), little is yet known about that of *H. hypnale* venom. In this chapter, some preliminary findings on the biochemical and molecular aspects of the major TLE isolated from this venom were reported.

11.2 METHODS

11.2.1 Isolation of TLE from *Hypnale hypnale* venom

Two milligram of *H. hypnale* venom was dissolved in 0.1% TFA and injected into a LiChrospher[®] RP100 C18 reverse phase column (250 x 4 mm, 5 μ m particle size) pre-equilibrated with 0.1% TFA. Elution was carried out at 1 ml/min flow rate with a linear gradient of 0.1% TFA in 100% acetonitrile over 60 min. The solvent delivery and gradient formation was achieved using the Shimadzu LC-20AD HPLC system. Eluted fractions were collected manually according to 215 nm absorbance, and dried in a Speed-Vac system. The fractions were reconstituted in 200 μ L PBS (pH 7.2) and screened for procoagulant activity using bovine fibrinogen (2 g/L) as described in Chapter 4 (Section 4.2.5.2).

The fraction which showed fibrinogen-clotting activity was further separated on a Superdex[™] 200 HR 10/30 gel filtration column (10 \times 300 mm, 13 μ m particle size; GE Healthcare, Sweden). Elution buffer was PBS (pH 7.2) at a flow rate of 0.75 ml/min. Protein was monitored by absorbance measurement at 280 nm and collected cautiously at small volume (0.3-0.5 ml in each tube). The column was calibrated using the following protein standards obtained from Bio-Rad (BIO-RAD Gel filtration Standard): thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). Fractionated products were screened for procoagulant activity using bovine fibrinogen (2 g/L), and the purity of protein was monitored by a 12% SDS-PAGE under reducing condition with beta-mercaptoethanol.

11.2.2 Deglycosylation of thrombin-like enzyme

The purified thrombin-like enzyme (1 mg/mL in PBS, pH 7.2) was incubated with *N*-glycosidase F (2 units/mg protein; Sigma) at 37 °C for 24 h and the deglycosylated protein was subjected to 12% SDS-PAGE under reducing condition.

11.2.3 Peptide sequencing and protein identification

The Coomassie Blue-stained gel band of purified thrombin-like enzyme was excised and subsequently processed at Proteomic International, Perth, Australia for in-gel trypsin digestion and MALDI-TOF/TOF analysis with peptide mass fingerprinting according to Bringans *et al.* (2008) (Section 10.3.2). The purified protein was also blotted onto a PVDF membrane by using Invitrogen iBlot™ System (Program P2, 8-10 minutes) and subjected to N-terminal sequencing by Edman degradation (Procise Sequencer, Applied Biosystem, available at Mission Biotech, Taipei, Taiwan ROC). The N-terminal sequence was compared to those of several snake venom thrombin-like enzymes. The internal and N-terminal sequences determined were also subjected to a multiple sequence alignment using progressive pair-wise alignments with hierarchical clustering (Corpet, 1988) to identify homologous regions in the peptides. Alignment was done using Mutalin® on-line software available from <http://multalin.toulouse.inra.fr/multalin/multalin.html>.

11.2.4 Arginine esterase activity and kinetic study

Arginine esterase was assayed using α -benzoyl arginine ethyl ester (BAEE) as substrate (Collins and Jones, 1972). The assay mixture contained 0.95 ml of 0.1-2.0 mM substrate in 0.05 M Tris-HCl buffer, pH 7.8, and 50 μ L of the thrombin-like enzyme (1 μ g). The

reaction was followed by measuring the rate of increase in absorbance at 255 nm. The difference in the extinction coefficient at 255 nm is $815 \text{ cm}^{-1}\text{M}^{-1}$.

11.2.5 Arginine amidase activity and kinetic study

Arginine amidase was determined according to Svendsen and Stocker (1977). Four tripeptide-substrates were used to investigate the TLE specificity. Each substrate was dissolved in 1:1 acetonitrile/water mixture. The arginine amidase activity was then determined by mixing 0.85 ml of 0.05 M Tris buffer (pH 8.0) with 100 μL of substrate (which final concentration was kept at 0.1-1.0 mM) and 50 μL of the thrombin-like enzyme (1 μg). The reaction was followed by measuring the absorbance at 410 nm, with extinction coefficient of $9400 \text{ cm}^{-1}\text{M}^{-1}$.

11.2.6 Fibrinogen clotting (procoagulant) activity on various fibrinogens

Procoagulant activity on various fibrinogens was determined by the method described by Bogarín *et al.* (2000). The thrombin-like enzyme (50 μg in 0.1 mL PBS) was added to 0.2 mL of fibrinogen solution (2 g/L) of various mammals at 37 °C. The coagulation time (time taken for fibrin strands to form) was recorded. A shorter coagulation time suggests a higher procoagulant activity.

To study the time course of the enzyme procoagulant activity, the thrombin-like enzyme (50 μg in 0.1 ml) was added to 0.2 ml of fibrinogen solution (2 g/l) preincubated at 37 °C. The absorbance of the mixture at 450 nm was read up to 50 minutes to monitor the formation and degradation of fibrin clots. The method was modified from Pirkle *et al.* (1986).

11.2.7 Fibrinogenolytic activity

Thrombin-like enzyme (10 µg) was added to a series of 1% bovine fibrinogen at the ratio of enzyme:substrate = 1:100 (w/w), followed by incubation at 37 °C. At indicated times (1 min, 1 h, 3 h, 6 h, 12 h and 24 h), the reaction was terminated by heating in a boiling water bath for 5 min, which also deproteinized the mixture. Clots that formed were removed by centrifugation at 10,000 g for 10 min. The supernatants containing fibrinopeptides were analyzed by reversed-phase HPLC. The solution (100 µL) was loaded onto a LiChrospher[®] RP100 C18 reverse phase column (250 x 4 mm, 5 µm particle size; Merck) on the Shimadzu LC-20AD HPLC system, and the fibrinopeptides A and B released from the bovine fibrinogen were characterized essentially as described by Shimokawa and Takahashi (1995). The eluents consisted of solvent A (0.025 M ammonium acetate, pH 6.0) and solvent B (50% acetonitrile in 0.05 M ammonium acetate, pH 6.0), with a linear gradient containing 0-100 % solvent B delivered over 60 min. The peptide peaks were monitored by measuring the absorbance at 214 nm.

A mixture of 1% bovine fibrinogen with 0.25 units of bovine thrombin was used as the positive control to exhibit the release of fibrinogen A and fibrinogen B. The negative control consisted of similar amounts of fibrinogen and denatured thrombin (by boiling prior to mixing for reaction). In another set of independent experiments, 20 µg of *H. hypnale* whole venom was used to react with the bovine fibrinogen to study the fibrinopeptide release at 24 h incubation.

11.3 RESULTS

11.3.1 Isolation and purification of thrombin-like enzyme (TLE)

Fibrinogen-clotting activity was detected in a heterogeneous fraction eluted between 40-45% ACN on reverse-phase HPLC. Further separation by gel filtration column yielded a fraction of procoagulant enzyme with molecular weight of approx. 35-40 kDa. On SDS-PAGE, the molecular weight was determined to be 37 kDa, while that of its deglycosylated form was 30 kDa, indicating that the enzyme was ~23% glycosylated in native condition (Figure 11.1).

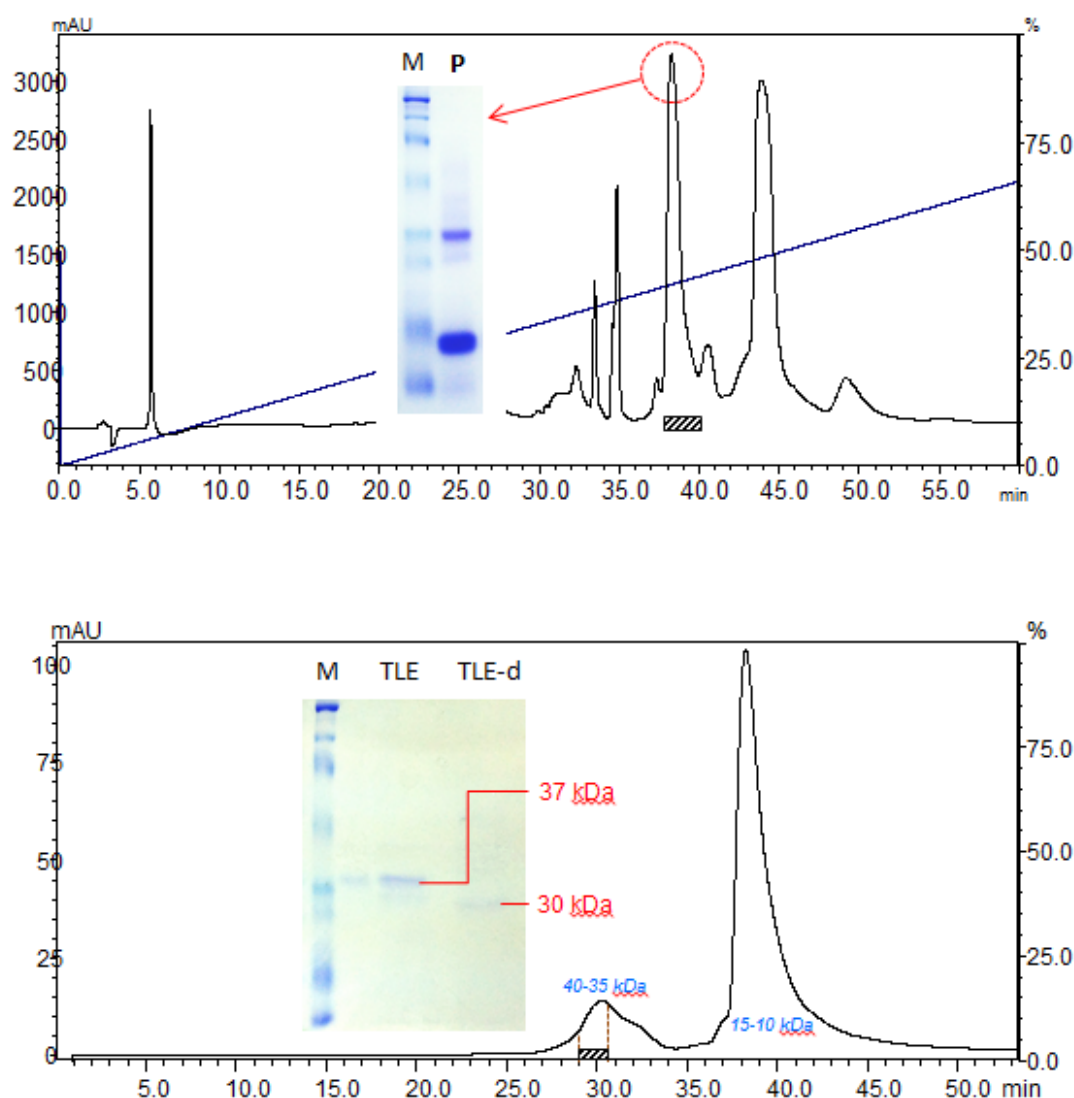


Figure 11.1 Purification of thrombin-like enzyme from *Hypnale hypnale* venom.

Upper panel: Fractionation of *H. hypnale* venom by reverse-phase HPLC monitored at absorbance 215 nm; lower panel: Separation of the procoagulant fraction by gel filtration HPLC monitored at absorbance 280 nm. Shaded bars at the base of a peak indicate the presence of fibrinogen-clotting activity. Insets showed the SDS-PAGE (under reducing condition) for the fractions of interest collected from the respective HPLC. M: molecular marker; P: procoagulant fraction; TLE: thrombin-like enzyme; TLE-d: deglycosylated thrombin-like enzyme.

11.3.2 Peptide sequencing and protein identification

Peptide mass finger printing of the 37 kDa thrombin-like enzyme identified it as a snake venom serine protease with high homologous internal sequences matched to thrombin-like enzymes ancrod (P26324) of *Calloselasma rhodostoma* (score: 51) and kangshuanmei (P85109) of *Gloydius brevicaudus* (score: 32) [Individual ions scores > 24 indicate identity or extensive homology ($p < 0.05$)]. The TLE isolated from *H. hypnale* venom was designated in the current work as hypnobin. The N-terminal sequence of hypnobin was also determined and compared with those of several procoagulant serine proteases in Table 11.1. In addition, hypnobin N-terminal and internal sequences were aligned with the sequences of some snake venom serine proteases in Figure 11.2 to show the regions of high homology consensus.

Table 11.1 N-terminal amino acid sequences of hypnobin and other serine proteases.

Enzymes	N-terminal sequences		References
	1	10	
Hypnobin	V I G G D E C N I N		This work
Ancrod	V I G G D E C N I N		Burkhart <i>et al.</i> (1992)
Kangshuanmei	V I G G D E C N I N		Sakai <i>et al.</i> (2006)
Acutobin	V I G G V E C D I N		Wang <i>et al.</i> (2001)
Purpurase	V V G G D E C N I N		Tan (2010)
Flavoxobin	V I G G D E C D I N		Shieh <i>et al.</i> (1988)
Crotalase	V I G G D E C N I N		Pirkle <i>et al.</i> (1981)
Gabonase	V V G G A E C K I D		Pirkle <i>et al.</i> (1986)
Kallikrein	V V G G Y N C E M N		Swift <i>et al.</i> (1982)
Trypsin	I V G G Y T C G A N		Titani <i>et al.</i> (1975)
Thrombin	I V E G Q D A E V G		Magnusson <i>et al.</i> (1975)

** Letters in red contribute to the homology of N-terminal sequences.

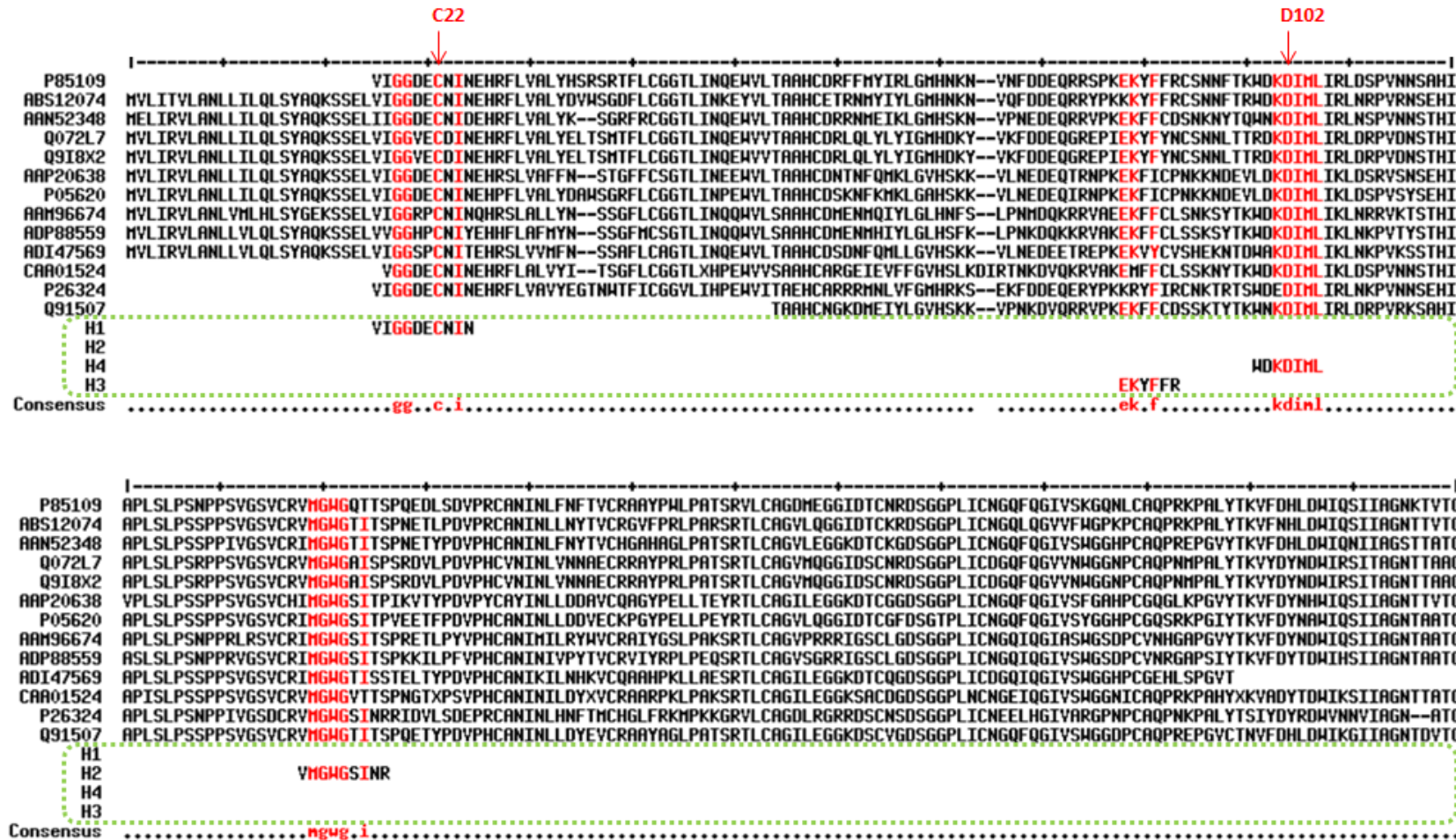


Figure 11.2 Alignment of the peptide sequence read by hypnobin with serine protease sequences from several Viperidae snakes.

(Figure 11.2, continued.)

H1: terminal sequence, H2-4: internal sequences of hypnabin; P85109 kangshuanmei (*Gloydius brevicaudus*); ADI47569 Serine protease (*Echis coloratus*); CAA01524 Ancrod-like protein (*Calloselasma rhodostoma*); AAM96674 Serine alpha-fibrinogenase precursor (*Macrovipera lebetina*); ADP88559 Serine alpha-fibrinogenase-like protein (*Daboia russellii siamensis*); ABS12074 Thrombin-like serine protease 1 (*Cryptelytrops albolabris*); Q91507 Mucrofibrase-1precursor (*Protobothrops mucrosquamatus*); AAN52348 stejnefibrase 1 (*Viridovipera stejnegeri*); Q072L7 Thrombin-like enzyme acutobin (*Deinagkistrodon acutus*); P05620 Thrombin-like enzyme flavoxobin (*Trimeresurus flavoviridis*); P26324 Thrombin-like enzyme ancrod (*Calloselasma rhodostoma*). Consensus regions are shown in red and these include the conserved cysteine 22 for disulfide bonding and aspartic acid 102 which forms the catalytic triad of serine proteases. Numbering was based on chymotrypsin. Alignment was done using Mutalin[®] on-line software available from <http://multalin.toulouse.inra.fr/multalin/multalin.html>.

11.3.3 Arginine esterase and amidase activities with kinetic studies

The K_m and k_{cat} values of hypnobin with BAEE as the substrate were 0.13 mM and 6.39 s⁻¹, respectively, giving rise to a k_{cat}/K_m of 49.15 mM⁻¹s⁻¹. The kinetic parameters of hypnobin on the tripeptide nitroanilide chromogenic substrates are shown in Table 11.2.

Table 11.2 Kinetic parameters of hypnobin with tripeptide nitroanilide substrates.

Substrate	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
N-benzoyl-L-proline-L-phenylalanine-L-arginine p-nitroanilide (BPPAN)	0.44	4.73	10.79
N-p-tosyl-glycine-L-proline-L-lysine p-nitroanilide (TGPLN)	0.69	5.59	8.10
N-benzoyl-L-phenylalanine-L-valyl-L-arginine p-nitroanilide (BPVAN)	0.21	1.46	6.78
N-benzyol-L-valine-L-glycine-L-arginine p-nitroanilide (BVGAN)	6.20	0.90	0.14

K_m : Michaelis constant. It is the substrate concentration at which the reaction rate is at half-maximum, and is an inverse measure of the substrate's affinity for the enzyme.

k_{cat} : Catalytic constant, or turnover number. It represents the number of reactions catalysed per unit time by each active site.

k_{cat}/K_m : This value reflects the enzyme efficiency. It indicates the number of 'turn overs' (reaction steps) at each active site per unit time by the enzyme.

11.3.4 Fibrinogen clotting (procoagulant) activity

Hypnobin exhibited rather distinct species-specific clotting activity on fibrinogens from various mammals (Table 11.3). The clotting time by hypnobin was in the decreasing order (i.e. increasing rate): rabbit << horse, goat < cow < human.

Table 11.3 The clotting time of hypnobin and bovine thrombin on various mammalian fibrinogens.

Mammalian origin of the fibrinogen	Hypnobin clotting time* (s)	Bovine thrombin clotting time (s)
Cow	177.7 ± 1.2	179.0 ± 0.6
Goat	195.3 ± 2.9	174.7 ± 1.8
Horse	245.3 ± 2.9	208.3 ± 2.7
Rabbit	Turbidity** > 300	Turbidity > 250
Human	167.7 ± 1.5	172.7 ± 1.5

The fibrinogen clotting time was determined at 37 °C by mixing 200 µg fibrinogen (2 mg/mL in PBS) and 1 µg hypnobin and 0.25 unit of bovine thrombin (amount of thrombin that causes similar clotting time on bovine fibrinogen with 1 µg hypnobin).

* Clotting time is defined as the time (s) for apparent fibrin strands to appear in the mixture of venom and fibrinogen.

** Turbidity refers to cloudiness appearing in the mixture of venom and fibrinogen, which may be interpreted as the formation of fibrin clots. Results are expressed as the mean ± SEM from three experiments.

In the time course study of hypnobin procoagulant activity (Figure 11.3), clotting was noted to be the least effective for rabbit fibrinogen and followed by horse fibrinogen: it was characterized by a slow increase in the clotting rate (defined by the slope/gradient of each curve calculated from the steepest upward portion of the graph), and a low amount of product as indicated by the maximal absorbance values. Clotting was fastest for the fibrinogen of human closely followed by that of cow, and then goat, all with similar product yields.

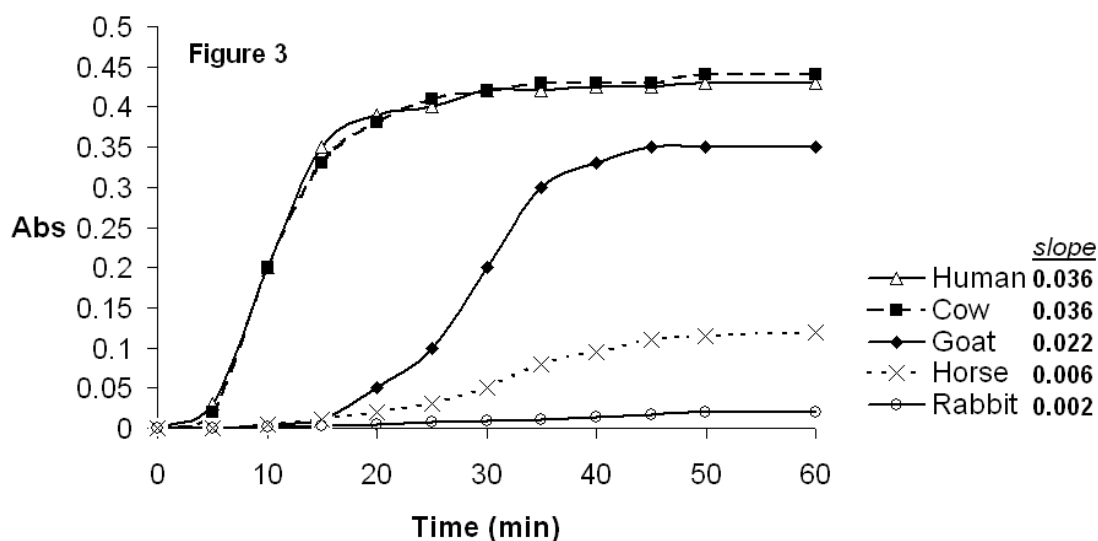


Figure 11.3 Time course of clotting activities of hypnobin on fibrinogens of several mammalian sources. The slope (gradient) for each curve is calculated from the steepest upward portion of the graph representing the maximal clotting rate expressed as absorbance/minute. Absorbance at 450 nm reflects the change in mixture clarity, which is responsible for the formation of fibrin strands as well as their dissolution/degradation.

11.3.5 Fibrinogenolytic activity

The reverse-phase chromatograms of the incubation mixtures of hypnobin and bovine fibrinogen were shown in Figure 11.4b-g, depicting the time-dependent fibrinopeptides-releasing by the TLE. Classically, as shown in Fig. 11.4a, bovine thrombin released both fibrinopeptides A and B, with the former released faster than the latter in the beginning of the reaction, but soon achieved an equal quantity (indicated by area under the peaks) by 10 min incubation. The peaks with retention times of 30.5 min and 33.5 min corresponded with the release of fibrinopeptides A and B, respectively. Hypnobin released fibrinopeptide A from the fibrinogen slower, with the fibrinopeptide A only detected from the incubation after 1 h reaction. The fibrinopeptide A level increased slowly since then over the next 23 h, and achieved its maximum at the end of the study period (24 h). A relatively much smaller amount of fibrinopeptide B was present at 6 h and persisted throughout the experiment ($\leq 10\%$ of fibrinopeptide A at 24 h). Under the same experimental condition, *H. hypnale* whole venom appeared to liberate both fibrinopeptides A and B from the bovine fibrinogen from the 24 h incubation (Figure 11.4h). However, the observation was accompanied by a slightly early shift in the retention time of fibrinopeptide B noted in repeated experiments.

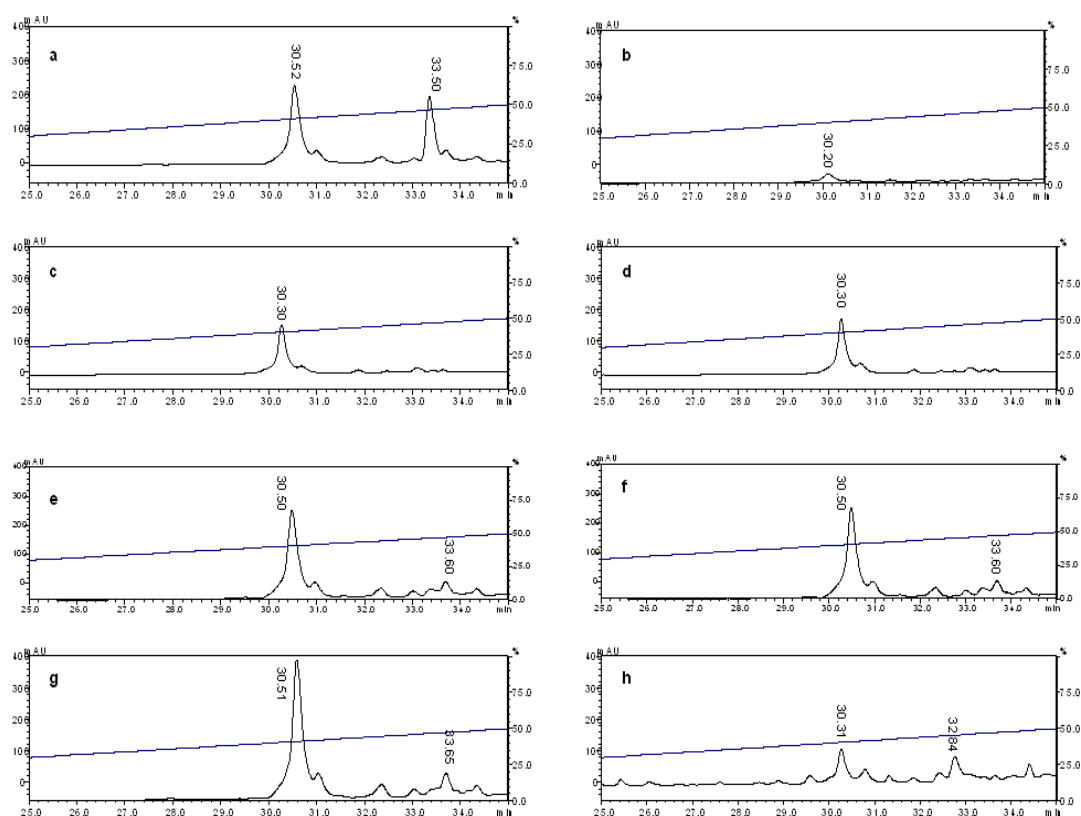


Figure 11.4 Reverse-phase HPLC profiles of fibrinopeptides release from incubation of bovine fibrinogen with (a) thrombin; (b)-(g) hypnobin; and (h) *Hypnale hypnale* venom. The incubation time of fibrinogen with thrombin and *H. hypnale* venom was 10 min and 24 h respectively, while that for hypnobin were 1, 2, 4, 6, 12 and 24 h (b-g, correspondingly). Major peaks observed reflected the cleavages of fibrinopeptide A and fibrinopeptide B by thrombin and thrombin-like enzyme, which are important mechanisms in the formation of fibrin clot.

11.4 Discussion

11.4.1 Purification and peptide sequencing of hypnabin

Defining thrombin-like enzymes from the myriad snake venom serine proteases can be challenging especially so when the term ‘thrombin-like’ is already rather a misnomer (Mackessy, 2010b). The current study undertook the approaches that cover salient physical, biochemical and molecular aspects for characterizing hypnabin with relevance to its ‘thrombin-like’ properties as conventionally suggested. Hypnabin appeared readily purified with a two-step chromatography approach, similar to previous reports for pure TLE isolation, e.g. purpurase from *Cryptelytrops purpureomaculatus* that involved a combination of Resource[®] Q anion exchanger and Superose[™] filtration column (Tan, 2010). Most of the TLE isolations appear somewhat technically demanding, likely because of the complex glycosylation nature of the enzyme. With its primary role for conferring stability to the enzymes, glycosylation has also given rise to multiple TLE isoforms, complicating the HPLC fractionation process. In this study, glycosylation of hypnabin was shown to be moderate and the molecular masses of both glycosylated and deglycosylated forms were within the expected range of most snake venom TLEs, excluding the likelihood of dimerization and extensive glycosylation where molecular weights could be as high as 50-70 kDa (Koh *et al.*, 2001; Paes Leme *et al.*, 2008). Nevertheless, the presence of other TLE isoforms within the venom remains possible and further exploratory isolation work should be performed.

The combined use of chromatography, gel electrophoresis and mass spectrometry has been advocated by several authors in proteomic studies of snake venoms (Li *et al.*, 2004; Calvete *et al.*, 2007b; Gutiérrez *et al.*, 2009), with the main objective to separate and

identify the proteins in venom on molecular basis. Some of the proteomic strategies were used in the study of hypnabin, where the purified protein from successive HPLC fractionation (showing single electrophoretic band) was tryptically digested into peptides and subjected to MALDI-TOF/TOF analysis and database search. Despite the lack of transcriptomic information of *H. hypnale* venom gland, the peptide sequences of the snake venom serine proteases (and thrombin-like enzymes) appeared to be highly homologous across taxa, therefore enabling the peptide matching possible for the identification of hypnabin. Noteworthy are the conserved cysteine 22 for disulfide bonding, and aspartic acid 102 which forms the catalytic triad of serine proteases. It reflects the role of balancing selection and gene duplication that have resulted in a robust conservation of serine protease structures across taxonomic levels (Calvete *et al.*, 2007b). In addition, the N-terminal amino acids of hypnabin was sequenced (a conventional method for protein identification), and the findings too revealed high degree of homology among the various crotalid venom TLEs from both New World and Old World species.

11.4.2 Arginine esterase and amidase activities with kinetic studies

Hypnabin, similar to many TLE of viperid venoms, exhibits esterolytic activity towards *N*- α -benzoyl-*L*-arginine ethyl ester, hence indicating its specific binding to the arginine residues in its substrate. Preferential hydrolysis was noted for thrombin specific substrates Bz-Phe-Val-Arg-pNA and Bz-Pro-Phe-Arg-pNA as indicated by the higher k_{cat}/K_m values. Interestingly, hypnabin also hydrolyzed p-Tos-Gly-Pro-Lys-pNA (a plasmin substrate) with activity comparable to that of the thrombin substrates. On the other hand, Bz-Val-Gly-Arg-pNA, a substrate for factor Xa, showed very minimal reaction with hypnabin, indicating its lack of activity on the activation of prothrombin.

11.4.3 Fibrinogen clotting activities

Snake venom TLEs were known to exhibit distinct specificity towards fibrinogens from different animals (Wik *et al.*, 1972; Tan, 2010). The observation was reported too in Chapter 4 where crude *H. hypnale* venom was used in the clotting reaction, and in the current experiment using the isolated TLE. These findings reflected the differences of fibrinogen structures from different animals as well as the specificity of snake venom TLE towards them. When monitored spectrophotometrically, the time course of fibrin clot assembly or dissolution induced by hypnokin could be described as: Generally, a higher clotting rate was accompanied by a shorter lag phase between the addition of enzyme and the first appearance of any turbidity, and the clot assembly appeared to persist with no dissolution within the measured time. The result seemed to suggest that hypnokin has a high affinity towards human fibrinogen with a rapid onset of procoagulant activity and prominent clot formation. This observation when extrapolated to clinical situation, it may reflect the intense turnover activity of the clotting enzyme that leads to rapid defibrinogenation and subsequent consumptive coagulopathy. The clotting time course profile of TLEs on various fibrinogens, however, should be distinguished from that of the whole venom where differences in clotting rates and early clot dissolution were noted (Section 4.3.7), presumably due to the presence of other enzymes in the venom that contributed to the observed effect. In this study, the finding that hypnokin reacted least towards rabbit fibrinogen is unequivocally consistent with the findings from reptilase, purpurase and albolabrase (Wik *et al.*, 1972; Tan, 2010; Tan *et al.*, 2012), as reported by Blomback *et al.* (1966), rabbit fibrinogen has a different amino acid composition from those of other animals.

11.4.4 Fibrinogenolytic activity

A limiting definition for a venom TLE includes the enzyme specific catalytic activity towards the A α and/or B β chains of fibrinogen, and its clotting mechanism mediated through the release of fibrinopeptides A (FpA) and/or B (FpB) (Mackessy, 2010b). Unlike thrombin, some venom TLEs release FpA predominantly, e.g. arvin (ancrod), flavoxobin and purpurase (Holleman and Coen, 1970; Shieh *et al.*, 1988; Tan, 2010) to trigger the clotting process. However, clotting with many venom TLEs is often accompanied by a later release of FpB, for examples, gabonase and cerastobin (Pirkle *et al.*, 1986; Farid *et al.*, 1989); whereas okinaxobin I almost exclusively releases FpB (Iwasaki *et al.*, 1990). Hence TLEs are broadly grouped based on their cleavage specificity for releasing the fibrinopeptides, although such specificity for the A α and/or B β chains is not absolute since there is substantial degradation of the alternate chain with increasing time (Markland, 1998b). The current study indicates that hypnabin preferentially releases FpA to initiate fibrinogen clotting. Upon extended enzyme reaction, digestion of fibrinogen B β chain was detected and it was assumed to be of limited activity, evidenced by the slow release of FpB with small amount, saturated at \leq 10% of the FpA maximum. This suggests that hypnabin belongs to the Class AB of thrombin-like snake venom serine proteases, along with the following TLEs from the venoms of Asiatic crotalids: acuthrombins from *Agkistrodon (Deinagkistrodon) acutus* (Huang *et al.*, 1999), kangshuanmei from *Agkistrodon (Gloydius) halys brevicaudus stejneger* (Zhang *et al.*, 2001), okinaxobin II from *Trimeresurus (Ovophis) okinavensis* (Nose *et al.*, 1994), a TLE from *Agkistrodon (Gloydius) saxatilis* (Koh *et al.*, 2001) and albolabrase from *Cryptelytrops albolabris* (Tan *et al.*, 2012), but not arvin (ancrod) from its close sister taxon, *Calloselasma rhodostoma*, which releases only FpA from fibrinogen (Holleman and Coen, 1970). The role of phylogeny and/or geo-trophic

adaptation is beyond the scope of this study for explaining such variations. Nonetheless, the finding indicates that hypnabin and ancrod likely possess different structural and functional determinants in terms of fibrinopeptide specificity; however, such difference does not seem to have any remarkable influence affecting the cross-neutralization of *H. hypnale* venom by the *C. rhodostoma* antivenom (Chapter 5). A possible explanation is that the common antigenic epitopes shared by the two TLE molecules extend beyond their catalytic domains for the cleavage of fibrinogen chain.

The identities of small peaks eluted between the FpA and FpB at extended reaction time are not known, although they may be related to other fibrinopeptide variants e.g. disarginine FpB that presents in small amounts (Koehn and Canfield, 1981). When incubated with fibrinogen, *H. hypnale* whole venom liberated both FpA and, to a lesser extent, FpB, an observation consistent with that of hypnabin. The release of FpA and FpB also suggests the possible coexistence of other β chain-selective TLE or β -fibrinogenase in the venom. The shift in the elution time of FpB (~1 min earlier) was probably due to some subtle modifications in the FpB peptide caused by coexistent active components in the venom.

It is known that incomplete release of FpA and FpB (as exhibited by the fibrinogenolytic mechanism of virtually all snake venom TLEs including hypnabin, which releases predominantly FpA followed by FpB slowly in lesser amount) forms fibrin clot that is friable and unstable. The intravascular coagulation affects the equilibrium of *in vivo* fibrinolytic system and triggers off plasmin activity (Collen, 1980). “Tenuous thrombi” or “weak clots” induced by the described mechanism are usually more susceptible to lytic degradation by endogenous plasmin (Turpie *et al.*,

1971), while potential fibrinolytic enzymes found in the venom may serve to augment the effect. Repeated formation and continuous dissolution of the tenuous thrombi will give rise to increased fibrin degradation products during the initial defibrinogenation phase, which may gradually normalize after fibrinogen depletion. In addition, certain fibrino(geno)lytic enzymes in some venom may potentiate the defibrinogenation effect by direct degradation of fibrin polymer (Siigur *et al.*, 1998) or by the generation of fibrinogenolytic products that are no longer convertible to normal fibrin clots by thrombin (Matsui *et al.*, 1998). This eventually results in hemostatic dysfunction associated with consumptive coagulopathy commonly reported in viperid snakebites, including *H. hypnale* envenomation (Premawardena *et al.*, 1996, 1998; Ariaratnam *et al.*, 2008). Further experimental characterization of the TLE is therefore essential not only for elucidating the functional mechanism of the protease in relation to its envenoming effect, but also for discovery of therapeutic candidates especially those related to the treatment of thromboembolic disorders.

CHAPTER 12

Conclusion

12.1 Present investigations: toxinological profiling of *Hypnale hypnale* venom

Important findings from the current investigations can be summarized in the following concluding remarks:

1. *Hypnale hypnale* venom exhibits a variety of enzymatic activities, noteworthy of the procoagulant enzymes, proteolytic enzymes, L-amino acid oxidases and phospholipases A₂. These are enzymes of moderate to high molecular masses present in most viperid venoms, and are significant in the pathophysiology of envenomations. Thrombin-like enzymes are related to the procoagulant activity which induces defibrinogenation and consumptive coagulopathy. Proteolytic enzymes contribute to systemic and local hemorrhagic syndrome. Some proteolytic enzymes and cytotoxic components such as L-amino acid oxidases and phospholipases A₂, induce massive cell deaths at the bite site and leading to tissue necrosis.
2. The median lethal dose (LD₅₀) of the venom varies on different routes of administration, in the increasing order of intravenous < intraperitoneal < intramuscular. The procoagulant nature of the venom possibly induces acute thromboembolic event and death when a substantial amount was administered intravenously. Intraperitoneal administration subjects the venom to first pass effect where venom proteins are likely degraded by hepatic metabolism. On intramuscular route, intense interaction of the venom proteins with local tissues leads to massive destruction of local tissues (including vasculature) and hence impairing the venom absorption. A higher intramuscular dose is therefore needed to cause systemic complications (principally hematoxic effects) and death.

3. The lethal, procoagulant, hemorrhagic and necrotic effects of *H. hypnale* venom were effectively cross-neutralized by the monovalent Malayan pit viper antivenom and Hemato polyvalent antivenom, with the polyvalent one being more superior. This is likely due to the presence of common antigenic properties between the venoms of *H. hypnale* and *Calloselasma rhodostoma* (Malayan pit viper) which share close phylogenetic relationship. The findings imply that Hemato polyvalent antivenom (which also cross-neutralized the lethal effect of Sri Lankan *Daboia russelii* venom) may be a beneficial therapeutic antivenom in the endemic areas where there is still no effective antivenom available clinically. Nevertheless, more extensive studies on various factors modulating the antivenom effectiveness may be required using suitable animal models, and the antivenom neutralization efficacy in humans needs to be assessed by clinical trials.
4. Acute kidney injury (AKI) was induced in mice experimentally envenomed with *H. hypnale* venom at 1 and 1.5 LD₅₀, characterized by raised blood urea and creatinine. Prolonged blood clotting time and hemorrhagic spots in lung implied bleeding tendency. Pallor noted in renal cortices was suggestive of renal ischemia secondary to consumptive coagulopathy. Intravenous infusion of Hemato polyvalent antivenom following the experimental envenomation effectively prevented death and AKI in all mice, hence supporting its potential therapeutic use in envenomation by *H. hypnale*.
5. Repeated immunization with *H. hypnale* venom in rabbits induced satisfactory antibody production. The antibody (prepared with capric acid precipitation method) was shown with comparable potency of venom neutralization (against lethal, procoagulant, hemorrhagic and necrotic activities) with the monovalent

Malayan pit viper antivenom, but less than that of Hemato polyvalent antivenom. The findings indicate the feasibility of producing an effective homologous antivenom against *H. hypnale* bite, as conceptually, a specific antivenom has a fuller neutralizing spectrum on various toxins in the homologous venom. Nevertheless, considering the extremely high costs associated with antivenom production, the socioeconomical status of the country, and the possibility of ambiguous diagnosis of the biting snake, it is proposed that the manufacturing of *H. hypnale* antivenom in the future be incorporated into the existing production of Indian polyvalent antivenoms (the current treatment of choice for envenoming by the Big Four in South Asia without addressing bites from *H. hypnale*)

6. Both the monovalent Malayan pit viper antivenom and Hemato polyvalent antivenom showed very high ELISA cross-reactivity (~90%) with *H. hypnale* venom, supporting the cross-neutralization activities reported. Using affinity-purified IgG raised against *H. hypnale* venom, the indirect ELISA showed that the IgG cross-reacted extensively with several Asiatic crotalid venoms, particularly that of *C. rhodostoma* (73.6%), indicating the presence of venom antigens common to both snakes. The levels of immunological cross-reactivity were vastly reduced with double-sandwich ELISA, and the assay was further shown to distinguish and quantify venoms of *H. hypnale*, *Daboia russelii* and *Echis carinatus sinhalensis* (three common local viperid), separately, in human sera spiked with the venoms. The assay hence can be a useful investigating tool for diagnosing biting species and studying the time course profile of venom concentrations in blood, especially during clinical studies.

7. Pharmacokinetic study in rabbits showed that *H. hypnale* venom has a long elimination half-life that is consistent with its prolonged systemic effects reported, and hence justifying the need for frequent and extended clinical monitoring on the coagulation profile and renal functions. The venom has an unusually low bioavailability (by intramuscular route), reflecting low systemic absorption from the injection site, possibly due to the venom's intense binding to the local tissues, while triggering destruction of the tissues and vasculatures that leads to compromised venom absorption. This may account for the huge difference of LD₅₀ between the intravenous and intramuscular routes. In experimentally envenomed rabbits, Hemato polyvalent antivenom altered the pharmacokinetics of *H. hypnale* venom with a remarkable initial blood venom level reduction followed by a subsequent rebound (venom redistribution phenomenon) following the law of mass diffusion. The redistributed venom was then completely neutralized with an additional dose of HPA. These findings were consistent with the pharmacological basis of an effective antivenom where immunocomplexes are formed to render the toxins inactive, while venom redistribution is essential to enhance venom toxins removal from tissues and hence a faster venom clearance from the body. The findings corroboratively support the previous cross-neutralization of Hemato polyvalent antivenom, and provide preliminary information for an optimization on the dosing and monitoring of the antivenom treatment.
8. Shotgun-LC-MS/MS approach identified a total of 52 proteins from *H. hypnale* venom of which 70% are secretory types and toxinologically related. The proteins however are restricted to only several major protein families, reflecting the recruitment of several physiological proteins and subsequent molecular adaptation under accelerated evolution that occurred in the venom glands over an

evolutionary time course. Several isoforms of toxic enzymes, e.g. snake venom metalloproteases, serine proteases, L-amino acid oxidases, are identified and likely possess diverse pharmacological properties as a result of molecular adaptation and neofunctionalization of the proteins. With the use of reverse-phase HPLC in studying the venom proteome, it was shown that the venom comprises of zinc-dependent metalloproteases, phospholipases A₂, L-amino acid oxidases, serine proteases and C-type lectins in high abundances. These major components in the venom correlate with the toxinological effects particularly on hematoxicity (hemorrhage, coagulopathy) and, local inflammation and tissue destruction (necrosis). The understanding of the venom composition is important in ascertaining the principal toxins, and for an optimization of antivenom formulation, for instance, to include both E6- and W6- subtypes of phospholipases A₂ in the spectrum of the antivenom neutralization.

9. A thrombin-like enzyme, hypnase, with molecular mass of 37 kDa (deglycosylated form: 30 kDa), was isolated from the venom with successive HPLC and shown to be homologous to several crotalid thrombin-like enzymes by comparing their internal and N-terminal sequences. It exhibits both arginine ester hydrolase and amidase activities, and has distinct specificity towards fibrinogens of different mammalian sources. In initiation of fibrinogen clotting, hypnase preferentially cleaves the A α chain of fibrinogen, therefore releasing fibrinopeptide A. Upon extended reaction time, a small amount of fibrinopeptide B is released, suggesting that hypnase belongs to the AB class of snake venom thrombin-like enzymes. The findings provide insights into the structures and functional mechanisms of the thrombin-like enzyme, and elucidate some aspects of the venom's hematoxic pathophysiology. In addition, the understanding of the

mechanism of this enzyme is important for potential drug discovery in the future, for example, as a drug lead of anticoagulant for thromboembolic disorders.

12.2 Future directions: further works suggested for *Hypnale hypnale* venom

There are several potential future research works related to *H. hypnale* venom. These suggested works are listed below:

1. Clinical trial of Hemato polyvalent antivenom in *H. hypnale* envenomations.
2. Clinical study of the sensitivity and specificity of the double-sandwich ELISA in detecting *H. hypnale* venom in blood samples from patients envenomed by the snake.
3. Study of the mechanisms of the venom nephrotoxic action, using isolated perfused rat kidney preparation, and an *in vivo* anesthetized rat model.
4. Purifications and characterizations of medically important components from the venom, e.g. zinc-dependent metalloproteases, phospholipases A₂, L-amino acid oxidases, C-type lectins etc.
5. Venom gland transcriptomic study for a solid and specific genetic database corresponding to the proteins synthesized in the gland.
6. Further study on the proteome and subproteome of *H. hypnale* venom using various state-of-the-art techniques.
7. Antivenomic study – based on the proteomic data, antivenom therapy can be analytically assessed *in vitro* by studying the extent of immunocomplexation with the principal toxins in the venom.
8. Comparative and neutralization studies of venoms from other *Hypnale* species (*H. nepa* and *H. zara*).

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Appendices

Appendix A. Ethics clearance letter for laboratory animal use



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PEJABAT KETUA

3 Mac 2010

Dr Fung Shin Yee
Jabatan Perubatan Molekul
Fakulti Perubatan
Universiti Malaya

Puan,

BIOMEDICAL AND TOXINOLOGICAL STUDIES ON SNAKE VENOM PROTEINS

Dengan sukacitanya Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan, Fakulti Perubatan, Universiti Malaya telah meluluskan permohonan untuk penyelidikan tersebut di atas.


No rujukan etika: **PM/03/03/2010/FSY (R)**

Sila ambil perhatian bahawa nombor rujukan etika yang diberi adalah sah untuk tempoh masa dua tahun sehingga **2 Mac 2012**.

Sila lengkapkan borang yang dilampirkan bersama dengan surat ini (Animal Traffic Record) dan hendaklah dikembalikan kepada pihak kami setelah penyelidikan tamat.





Sekian, terima kasih.

Yang benar,




Dr. Haji Azizuddin Bin Haji Kamaruddin
Ketua
Pusat Haiwan Makmal
Fakulti Perubatan
Merangkap Setiausaha Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan

SK : Puan Zura Syazleena Hamizan
Setiausaha MCRC
Pejabat Dekan
Fakulti Perubatan

MS ISO 9001:2000 REG. NO. AR 2760

Ketua, Pusat Haiwan Makmal
Fakulti Perubatan, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
Head, Laboratory Animal Centre
Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia
Tel: (603) 7967 4792 Faks: (603) 7955 9886
E-mail: azizud@um.edu.my Website: <http://www.um.edu.my>



Appendix B. List of proceedings and conference paper presentations

Tan CH, Sim SM, Tan NH, Fung SY, Ariatname CA, Ponnudurai G. (2010). Enzymatic and pharmacological activities of Merrem's humped-nose pit viper (*Hypnale hypnale*) venom and its neutralization by a commercial antivenom. 24th Scientific Meeting of the Malaysian Society of Pharmacology and Physiology, O119, pp.64. Shah Alam, Malaysia. (02-03 Jun 2010) [oral, with Best Award]

Tan Choo Hock, Tan Nget Hong, Sim Si Mui, Christeine Ariatnam, Gnanajothy Ponnudurai, Fung Shin Yee. (2010). Biochemical and toxinological characterisation of *Hypnale hypnale* pit viper venom and its neutralisation by a commercial antivenom. 35th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology, MSBMB and CARIF, Oral 8, pp.36. Bangi, Malaysia. (27-28 Jul 2010) [oral]

Choo-Hock Tan, Shin-Yee Fung, Gnanajothy Ponnudurai, Christeine A. Gnanathan, Si-Mui Sim and Nget-Hong Tan. (2010). Immune response elicited by immunization of rabbits with *Hypnale hypnale* venom and the ELISA cross-reactivities of the antibodies. Proceedings of the 4th International Conference on Natural Toxins, Oral A3, pp.82, Ismailia, Egypt. (20-22 December, 2010) [oral]

Tan NH, Tan CH, Fung SY, Ponnudurai G, Ariaratnam CA, Sim SM. (2010). The toxinological properties of Merrem's humped-nose pit viper (*Hypnale hypnale*) and its neutralization by commercial antivenoms, India National Toxin Conference, India. (01-02 Nov, 2010) [Prof Tan NH as invited speaker]

Choo-Hock Tan, Si-Mui Sim, Christeine A. Gnanathan, Shin-Yee Fung, Gnanajothy Ponnudurai, Nget-Hong Tan. (2011). Pharmacokinetics of the venom of hump-nosed pit viper (*Hypnale hypnale*) in rabbits. Proceedings of the 84th Annual Meeting of the Japanese Pharmacological Society and the 11th Southeast Asian Western Pacific Regional Meeting of Pharmacologists. In Journal of Pharmacological Sciences, 115(1), pp.235P. P2J23-3. Yokohama, Japan. (22-24 March, 2011) [poster]

Tan, C.H., Gnanathan, C.A., Sim, S.M., Fung, S.Y., Tan, N.H. (2011). Toxicological and immunological characterization of the Sri Lanka hump-nosed pit viper (*Hypnale hypnale*) venom, in: Constantine, G.R. (Ed.), University of Colombo Annual Research Symposium. (Proceedings of the 2011 Annual Research

Symposium of the University of Colombo, Colombo, Sri Lanka). Proceedings, p. 219-220. Colombo, Sri Lanka. (28-29 June, 2011) [Invited plenary lecture]

Gnanathan, C.A., Tan, C.H., Sim, S.M., Fung, S.Y., Tan, N.H. (2011). Toxicological and immunological characterization of the Sri Lanka hump-nosed pit viper (*Hypnale hypnale*) venom. 17th Congress of the European Section of the INTERNATIONAL SOCIETY on TOXINOLOGY. Program & Abstracts Book, Poster: 61, p. 153. Valencia, Spain. (11-15th September, 2011). [poster presented by Prof. Christine (Gnanathan C. A.)]

Choo-Hock Tan, Si-Mui Sim, Gnanathan Chirsteine Ariaratnam, Shin-Yee Fung, Nget-Hong Tan. (2011). ELISA in *Hypnale hypnale* Pit Viper Envenoming: An approach for diagnostic and therapeutic optimization. 4th Regional Conference on Molecular Medicine. Programme and Proceedings, p. 36. Penang, Malaysia. (9-11 October, 2011) [oral]

Tan Choo Hock, Sim Si Mui, Tan Nget Hong, Fung Shin Yee, Christine Ariaratnam Gnanathan. (2012). Profiling the venom of hump-nosed pit viper (*Hypnale hypnale*): Toward an optimization for treatment, diagnostics, monitoring and potential drug discovery. Post-graduate Research Day (in conjunction with 19th Scientific Meeting of MSMBB 2012). University of Malaya, Kuala Lumpur. (31 October, 2012) [oral, finalist contestant]

Tan Choo Hock. (2012). Toxinological Characterizations of the venom of hump-nosed pit viper (*Hypnale hypnale*). Three-Minute Thesis Contest (Ph.D). Faculty of Medicine, University of Malaya, Kuala Lumpur. (22 November, 2012) [Second Prize, Faculty level]

Appendix C. List and copies of publications in ISI-indexed journals

- Tan, C.H., Leong, P.K., Fung, S.Y., Sim, S.M., Ponnudurai, G., Ariaratnam, C., Khomvilai, S., Sitprija, V., & Tan, N.H. (2011). Cross neutralization of *Hypnale hypnale* (hump-nosed pit viper) venom by polyvalent and monovalent Malayan pit viper antivenoms in vitro and in a rodent model. *Acta Trop.*, 117(2), 119-124. doi: 10.1016/j.actatropica.2010.11.001
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Cross neutralization of *Hypnale hypnale* (hump-nosed pit viper) venom by polyvalent and monovalent Malayan pit viper antivenoms *in vitro* and in a rodent model

Choo Hock Tan^b, Poh Kuan Leong^b, Shin Yee Fung^a, Si Mui Sim^b, Gnanajothy Ponnudurai^c, Christeine Ariaratnam^c, Sumana Khomvilai^d, Visith Sitprija^d, Nget Hong Tan^{a,*}

^a Department of Molecular Medicine, Faculty of Medicine, CENAR, University of Malaya, 50603 Kuala Lumpur, Malaysia

^b Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

^c Department of Human Biology, International Medical University, 126 Jalan 19/155B, Bukit Jalil, Kuala Lumpur, Malaysia

^d Queen Saovabha Memorial Institute, Rama IV Road, Bangkok, Thailand

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ABSTRACT

Hypnale hypnale (hump-nosed pit viper) is a medically important venomous snake in Sri Lanka and Southwestern India. Bite of this snake may result in hemostatic dysfunction, acute kidney injury and death. Clinical studies indicated that the locally available polyvalent antivenoms produced in India are not effective against hump-nosed pit viper envenoming. Hence, there is an urgent need to search for effective antivenom. In this paper, we examined the ability of *Calloselasma rhodostoma* (Malayan pit viper) monovalent antivenom and the Hemato polyvalent antivenom (both produced by Thai Red Cross Society, TRCS) to neutralize the lethality and toxic effects of *H. hypnale* venom, as *C. rhodostoma* is considered a sister taxon of *H. hypnale*. *In vitro* neutralization studies showed that the Hemato polyvalent antivenom effectively neutralized the lethality of *H. hypnale* venom (1.52 mg venom/mL antivenom) as well as the hemorrhagic, procoagulant and necrotic activities of the venom. The monovalent *C. rhodostoma* antivenom could also neutralize the lethality and toxic activities of the venom, but the potency was lower. The Hemato polyvalent antivenom also effectively protected mice from the lethal and local effects of *H. hypnale* venom in an *in vivo* rodent model of envenoming. Furthermore, the polyvalent antivenom could also effectively neutralize the venom of *Daboia russelii* (2.50 mg venom/mL antivenom), another common cause of snake bites in Sri Lanka and South India. These findings suggested that the Hemato polyvalent antivenom may be beneficial in the antivenom treatment of *H. hypnale* envenoming.

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1. Introduction

Hypnale hypnale (hump-nosed pit viper) is widely distributed in Sri Lanka and southwestern coast of India (Ariaratnam et al., 2008). It is the major cause of venomous snake bites in Sri Lanka (de Silva et al., 1994). In India, the medical importance of *H. hypnale* bite has been overlooked for many years perhaps because of misidentification of biting species mainly as *Echis carinatus*. Recently, Joseph et al. (2007) reported the first authenticated cases of life-threatening envenoming by *H. hypnale* in southwestern India. de Silva et al. (1994) and Ariaratnam et al. (2008) also reported that bites by this snake can cause debilitating local and fatal systemic envenoming. Victims of *H. hypnale* bite may develop hemostatic dysfunctions including coagulopathy, thrombocytopenia or spontaneous systemic hemorrhage, as well as acute kidney injury, with an overall

fatality rate of 1.7% in hospitalized patients (Ariaratnam et al., 2008; Joseph et al., 2007; Premawardena et al., 1996, 1998).

Previous clinical and laboratory studies of *H. hypnale* venom demonstrated that the venom exhibits procoagulant and fibrinolytic activities (de Silva et al., 1994; Premawardena et al., 1998) and contains phospholipases A₂ similar to those in the Malayan pit viper (*Calloselasma rhodostoma*) venom (Wang et al., 1999). Tan et al. (2010) also reported the presence of hyaluronidase, L-amino acid oxidase, thrombin-like enzymes, arginine esterase and proteases in the venom. To date, however, there is still no specific antivenom against the venom for envenoming treatment. The polyvalent antivenoms available in Sri Lanka and southwestern India, including Bharat polyvalent antivenoms and Haffkine polyvalent antivenoms, for examples, were found to be not effective in the treatment of *H. hypnale* bite (Ariaratnam et al., 2008; Joseph et al., 2007). There is therefore an urgent need to search for effective antivenom against the venom.

The Malayan pit viper (*C. rhodostoma*) is the sister taxon of *H. hypnale* (Parkinson et al., 1997; Vidal and Lecointre, 1998). Previ-

* Corresponding author. Tel.: +60 3 79674912; fax: +60 3 79674957.

E-mail address: tanngethong@yahoo.com.sg (N.H. Tan).

ously, both snakes were classified as members of the *Agkistrodon* genus. As such, the venom of *C. rhodostoma* may possess toxic components similar to those in the *H. hypnale* venom. In view of the close phylogenetic relationship between *H. hypnale* and *C. rhodostoma*, the Thai Red Cross Malayan Pit Viper antivenom had been tested for neutralizing *H. hypnale* venom using rodent assay (Ariaratnam et al., 2008). However, there was no effective protection found. In this paper, we report our studies on the *in vitro* cross-neutralizations of *H. hypnale* venom by newly manufactured monovalent Malayan pit viper antivenom, and *in vitro* as well as *in vivo* (rodent model) cross-neutralizations of the venom by the newly developed Hemato polyvalent antivenom. Both antivenoms were produced by Thai Red Cross Society (TRCS) in Thailand. The Hemato polyvalent antivenom was produced by immunization of horses with a mixture of the following venoms, all of Thai origins: *C. rhodostoma*, *Cryptelytrops albolabris* (Green pit viper) and *Daboia siamensis* (Russell's viper).

2. Materials and methods

2.1. Venoms and antivenoms

H. hypnale venom was a pooled sample obtained from the milking of >10 adult snakes captured in Sri Lanka (Gamapha, Kelaniya, Avissawela, Colombo regions). The snakes were kept at the snake farm at University of Colombo, Sri Lanka and were identified by Ansem de Silva, an expert herpetologist. *Daboia russelii* venom was from the same source and the snakes were captured in Sri Lanka (Anuradhapura, Ratnapura, Galle and Colombo regions). *C. rhodostoma* (Malayan pit viper, from Malaysia) venom and *E. carinatus sochureki* (Pakistan) venom used in this study were obtained from Latoxan (France). Monovalent Malayan pit viper antivenom (MPVA) (Batch no. CR00909, exp. date 2/11/2014) and Hemato polyvalent antivenom (HPA) (Batch no. HP00108, exp. date 6/11/2013) were supplied by Queen Saobhva Memorial Institute, Thai Red Cross Society (TRCS). The antivenoms were freeze-dried $F(ab')_2$ (90%) immunoglobulin fragments, obtained from hyperimmunized horses, refined by caprylic acid precipitation and pepsin digestion. The Bharat Polyvalent ASVS (anti-snake venom serum) (Batch no. A5309049, exp. date 03/2013) is a gift from Bharat Serums and Vaccines, Mumbai, India. This antivenom is a refined and concentrated preparation of $F(ab')_2$ obtained by fractionating antisera from hyperimmunized horses, and is capable of neutralizing cobra, common krait, Russell's viper and saw-scaled viper venoms. The antivenoms were reconstituted with sterile water according to instructions and stored at 4 °C.

2.2. Reagents and animals

All reagents and chemicals were purchased from Sigma Chemical Company (USA) and were of analytical grade. Albino mice (ICR strain) were supplied by the Laboratory Animal Center, Faculty of Medicine, University of Malaya. The animals were handled according to the guidelines given by CIOMS on animal experimentation (Howard-Jones, 1995). Human citrated sera were a pooled sample from five healthy adult volunteers with consents.

2.3. Determination of lethality, hemorrhagic, procoagulant and necrotic activities

The LD₅₀ values of the venom were determined by intravenous (via caudal veins) as well as intramuscular injection into mice (16–25 g, $n=4$) and the survival ratio was recorded after 48 h. The LD₅₀ (and the 95% confidence intervals, C.I.) values were calculated with the probit analysis method of Finney (1952), using Biostat

Analysis software. MLD (minimum lethal dose) of the venom was the dose that killed all the animals tested.

Hemorrhagic activity was determined using mice, a method modified from Theakston and Reid (1983) where rats were used. The minimum hemorrhage dose (MHD) was determined by injecting 40 µL of varying amount of venom (dissolved in normal saline) intradermally into the shaved dorsal skin of lightly sedated mice (with diethyl ether). After 90 min, the animals ($n=3$ per dose) were killed by overdose of diethyl ether and the dorsal skin was removed. The mean diameter of the hemorrhagic lesion on the inner surface of the skin was measured immediately upon skin removal. Dose response curve between the mean diameter of the hemorrhagic lesion and the venom dose was plotted. The MHD was the dose that caused a hemorrhagic lesion diameter of 10 mm.

Procoagulant activity was determined by the method adapted from Bogarin et al. (2000). Various amounts of venom, dissolved in 0.1 mL of normal saline, were added separately into 0.2 mL of bovine fibrinogen solution (2 g/L) or 0.2 mL of human citrated plasma at 37 °C. Coagulation times were recorded. The minimum coagulant dose (MCD) is defined as the minimal amount of venom (µg/mL) that clots a standard solution of bovine fibrinogen or human citrated plasma in 60 s.

Necrotic activity was determined by injection of the venom at varying amount intradermally into the shaved dorsal skin of lightly sedated mice (with diethyl ether), and the animals were kept with free access to water and feed *ad libitum*. After 72 h, the animals ($n=3$) were killed by overdose of diethyl ether and the dorsal skin was removed. The mean diameter of the necrotic lesion was then measured immediately upon skin removal. The minimum necrotic dose (MND) is defined as the amount of venom that induces a necrotic lesion with a diameter of 5 mm.

2.4. *In vitro* neutralization of the venom lethality, hemorrhagic, procoagulant and necrotic activities by antivenoms

These were carried out as modified from Ramos-Cerrillo et al. (2008).

Neutralization of lethality: 5 LD₅₀ of *H. hypnale* venom was mixed thoroughly with various dilutions of the antivenom in phosphate-buffered saline, to give a total volume of 300 µL. The mixture was incubated at 37 °C for 30 min with gentle shaking. The mixture was subsequently centrifuged at 10,000 × *g* and then injected intravenously into the caudal vein of mice, and the number of animals survived 48 h post-injection was recorded. Neutralization potency of the antivenom is measured by ED₅₀ (µL antivenom/5 LD₅₀ venom), which is defined as the amount of antivenom (µL) at which the survival ratio of the animals is 50%. The estimated amount of venom neutralized per mL of antivenom was then calculated based on the ED₅₀ value, the LD₅₀ value and weight of animals used.

Neutralization of the hemorrhagic activity: 2 MHDs of *H. hypnale* venom was mixed thoroughly with various dilutions of the antivenom in phosphate-buffered saline, to give a total volume of 40 µL. The mixture was incubated at 37 °C for 30 min with gentle shaking. The mixtures were subsequently injected intradermally into the dorsal areas of lightly sedated mice (with diethyl ether). After 90 min, the animals were killed by overdose of diethyl ether. The dorsal skin was removed immediately and the mean diameter of the hemorrhagic spot was measured. Neutralization potency of the antivenom is measured by ED₅₀ which is defined as the volume of antivenom (µL) which reduced the activity of the challenge dose (2 MHDs) of the venom by 50%. For comparison purpose, it is also expressed in terms of the ratio of µL antivenom/mg venom.

Neutralization of procoagulant activity: 2 MCDs of *H. hypnale* venom were mixed thoroughly with various dilutions of the antivenom in phosphate-buffered saline, to give a total volume of

100 μ L. The mixture was incubated at 37 °C for 30 min with gentle shaking. To these, 200 μ L of bovine fibrinogen (2 g/L) or human citrated plasma, preincubated at 37 °C, was added and the coagulation times were recorded. Neutralization potency of the antivenom is measured by effective dose (ED), which is defined as the volume of the antivenom (μ L) at which coagulation time was increased three times when compared to coagulation time of the fibrinogen solution or citrated human plasma incubated with venom alone. For comparison purpose, it is also expressed in terms of the ratio of μ L antivenom/mg venom.

Neutralization of necrotic activity: 2.5 MNDs of the venom (100 μ g) was mixed well with various dilutions of the antivenoms in phosphate-buffered saline to give a total volume of 50–80 μ L. The mixtures were incubated at 37 °C for 30 min under gentle agitation. The mixtures were subsequently injected intradermally into the dorsal areas of lightly sedated mice (with diethyl ether), and the animals were kept with free access to feed and water *ad libitum*. After 72 h, the animals were killed by an overdose of diethyl ether and following which, the skins were removed immediately to examine the mean diameter of the dermal necrotic lesion. Neutralization potency of the antivenom is measured by ED₅₀ which is defined as the volume of antivenom (μ L) which reduced the necrotic activity of the challenge dose (2.5 MNDs) of the venom by 50%. For comparison purpose, it is also expressed in terms of the ratio of μ L antivenom/mg venom.

2.5. *In vivo* neutralization of the lethality of *H. hypnale* venom by Hemato polyvalent antivenom using a rodent model

This was carried out by intramuscular injection of the minimum lethal dose (20 μ g/g) of *H. hypnale* venom into mice ($n=4$) followed by intravenous injection of 200 μ L of the Hemato polyvalent antivenom, 5 min after the venom inoculation. The number of animals survived 48 h post-injection was recorded. The local effects of the venom were also examined. Control group consisted of mice ($n=4$) challenged with intramuscular minimum lethal dose of the venom, followed by intravenous injection of 200 μ L of normal saline.

2.6. Statistical analysis

Results for procoagulant, hemorrhagic and necrotic activities are presented as mean \pm S.E.M., while the variability of lethality assays was expressed as 95% confidence intervals (C.I.). The significance of the differences of the means was determined by Student's *t*-test. ED₅₀ (median effective dose) and the 95% confidence intervals (C.I.) were calculated using the probit analysis method of Finney (1952). Statistical analyses were carried out using the Biostat Analysis software.

3. Results and discussion

3.1. Toxinological activities of *H. hypnale* venom

The intravenous LD₅₀ and intramuscular LD₅₀ of *H. hypnale* venom were determined to be 0.90 μ g/g mouse (95% confidence interval of 0.42–1.84 μ g/g mouse) and 13.7 μ g/g (8.44–19.50 μ g/g mouse), respectively. The *i.v.* LD₅₀ reported herein is slightly lower than an earlier one reported by Ariaratnam et al. (2008), who reported an intravenous LD₅₀ of 65.4 μ g per mouse, or approximately equivalent to 3 μ g/g. The difference could be due to either geographic or individual variation. The *i.v.* LD₅₀ of the venom is comparable to that of *C. rhodostoma* venom examined in this study (LD₅₀ of 1.48 μ g/g, 95% C.I. 0.78–2.06 μ g/g mouse).

The venom exhibited strong procoagulant, hemorrhagic and necrotic activities. This is consistent with the clinical observations

in proven *H. hypnale* bite (Ariaratnam et al., 2008). The minimum coagulation dose (MCD) is 56.2 μ g/mL for bovine fibrinogen, and 55.1 μ g/mL for human citrated plasma. The minimum hemorrhagic dose (MHD) and minimum necrotic dose (MND) are 10.5 μ g and 39.3 μ g, respectively. These values are comparable to those of the *C. rhodostoma* venom, with MCDs of 27.3 μ g/mL for bovine fibrinogen or 24.9 μ g/mL for human citrated plasma; MHD of 24.0 μ g and MND of 28.7 μ g, respectively.

3.2. *In vitro* neutralization of *H. hypnale* and *C. rhodostoma* venoms

We examined the abilities of three commercial antivenoms to neutralize the toxic activities of *H. hypnale* venom *in vitro*. The neutralization was evaluated in assays involving incubation of venom and antivenom for 30 min prior to testing.

Bharat polyvalent antivenom (ASVS), the antivenom produced by immunizing horses with venoms from the 'Big 4' (*Naja naja*, *Bungarus caeruleus*, *D. russelii* and *E. carinatus*) failed to protect against *H. hypnale* venom: all mice ($n=4$) injected with 5 LD₅₀ (*i.v.*) of the venom died despite receiving 200 μ L/mouse of the antivenom. This is consistent with the clinical observations reported by Joseph et al. (2007) that administration of the Bharat polyvalent antivenom did not bring any benefits to patients envenomated by *H. hypnale*. Other authors also reported that the Haffkine polyvalent antivenom (also produced against the 'Big 4') was totally ineffective in the treatment of victims bitten by *H. hypnale* (Ariaratnam et al., 2008; Sellaheewa et al., 1995).

The capability of the monovalent Malayan pit viper (MPV) antivenom to neutralize *H. hypnale* venom was then examined, as Malayan pit viper (*C. rhodostoma*) is considered a sister taxon to *H. hypnale* by mitochondrial DNA analysis (Parkinson et al., 1997). Ariaratnam et al. (2008) reported that (expired) monovalent MPV antivenom failed to neutralize 5 *i.v.* LD₅₀ of *H. hypnale* venom *in vitro*, even with 200 μ L of the antivenom. A preliminary study, however, showed that when lesser amount of *H. hypnale* venom was used (2 LD₅₀, intraperitoneal injection), the newly manufactured monovalent MPV antivenom could neutralize the lethality of the venom in mice, though only moderately (Tan et al., 2010). The present study showed that the newly manufactured monovalent MPV antivenom indeed could effectively protect mice injected with 5 *i.v.* LD₅₀ of *H. hypnale* venom, and the ED₅₀ was determined to be 70.71 μ L/5 *i.v.* LD₅₀, or equivalent of 0.89 mg venom neutralized per mL of antivenom. The discrepancies with the previous report by Ariaratnam et al. (2008) could be due to batch differences in the antivenom, as the batch of monovalent MPV antivenom used in this study was manufactured using a new process (caprylic acid precipitation) and appears to be more potent (Khomvilai, 2008). The monovalent MPV antivenom was also effective in the neutralization of the procoagulant effect on both bovine fibrinogen and human citrated plasma, with EDs of 432.1 μ L/mg venom and 384.4 μ L/mg venom, respectively. It also neutralized the hemorrhagic and necrotic activities of *H. hypnale* venom, with ED₅₀s of 472.3 μ L/mg venom and 612.6 μ L/mg venom, respectively. As expected, the monovalent MPV antivenom was much more potent in the neutralization of the lethality and toxicities ($p<0.05$) of *C. rhodostoma* venom, in particular the necrotic and hemorrhagic activities. The ED₅₀ against *C. rhodostoma* venom lethal effect was 41.53 μ L/5 *i.v.* LD₅₀, or equivalent of 3.23 mg venom neutralized per mL of antivenom. The neutralization ED/ED₅₀s are 209.2 μ L/mg and 152.7 μ L/mg for procoagulant activities against bovine fibrinogen and human citrated plasma, respectively; 151.7 μ L/mg for hemorrhagic activity and 38.1 μ L/mg for necrotic activity of the venom (see Tables 1 and 2). Nevertheless, these results suggest that at least some of the venom toxins from the two snakes, *H. hypnale* and *C. rhodostoma*, are antigenically similar. It is known that the

Table 1
Neutralization of lethality of *Hypnale hypnale*, *Calloselasma rhodostoma* and other venoms by the monovalent Malayan pit viper antivenom and Hemato polyvalent antivenom.

Antivenom	Venom	LD ₅₀ (i.v.) (μg/g)	ED ₅₀ (μL antivenom/5 i.v. LD ₅₀)	mg venom neutralized per mL antivenom
Monovalent Malayan Pit Viper antivenom	<i>C. rhodostoma</i>	1.48 μg/g (0.78–2.06)	41.53 μL (20.4–88.4)	3.23
	<i>H. hypnale</i>	0.90 μg/g (0.42–1.84)	70.71 μL (33.7–148.4)	0.89
Hemato polyvalent antivenom	<i>C. rhodostoma</i>	1.48 μg/g (0.78–2.06)	22.47 μL (14.8–34.1)	7.14
	<i>H. hypnale</i>	0.90 μg/g (0.42–1.84)	41.53 μL (20.4–88.4)	1.52
	<i>D. russelii</i>	0.24 μg/g (0.19–0.62)	7.52 μL (3.53–15.3)	2.50
	<i>E. carinatus sochureki</i>	2.08 μg/g (1.02–4.42)	>200 μL	Not effective

Values in range for LD₅₀ and ED₅₀ indicated 95% confidence intervals. For neutralization experiments, mice ($n = 4$) were challenged with 5 i.v. LD₅₀ of the various venoms.

procoagulant enzymes (thrombin-like enzymes) and hemorrhagins from different venomous snakes can have very different antigenic properties. For example, thrombin-like enzyme from *Cryptelytrops purpureomaculatus* exhibited very little ELISA cross reactions with the thrombin-like enzymes from *C. rhodostoma* venom (Tan, 2010). Also, Fung (2002) reported that the major hemorrhagin of *C. purpureomaculatus* venom did not cross-react with *C. rhodostoma* venom at all when examined by double sandwich ELISA. The ability of the monovalent MPV antivenom to neutralize the lethality, procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom therefore supports the conclusion by Parkinson et al. (1997) that *C. rhodostoma* is a sister taxon to *H. hypnale*. This is an interesting example of similarities in the immunological properties of the venom proteins support phylogenetic relationship derived from mitochondrial DNA analysis. The close phylogenetic relationship between *H. hypnale*, found in India subcontinent and *C. rhodostoma*, found in Southeast Asia, is a fascinating example of the existence of the 'Malayan' element in the fauna of Peninsular India. This phenomenon has been known since a long time, and the cause of these similarities have been explained by the Satpura Hypothesis (Hora, 1949), according to which fauna migration followed the Satpura trends of mountain through Peninsular India during the Pleistocene. The validity of Satpura Hypothesis, however, has been disputed recently (Karanth, 2003).

Recently, a new polyvalent antivenom against hematotoxic snake venoms in Thailand has become available. This polyvalent antivenom, termed Hemato polyvalent antivenom, was produced from plasma of horses hyperimmunized by venoms from the three common viper and pit vipers in Thailand: Green pit viper (*C. albolabris*), Russell's viper (*D. siamensis*) and Malayan pit viper (*C. rhodostoma*). The Hemato polyvalent antivenom was found to be much more effective than the monovalent MPV antivenom in

neutralizing the lethality, procoagulant, hemorrhagic and necrotic activities of *C. rhodostoma* venom: the ED₅₀ for neutralization of the lethality was determined to be 22.47 μL/5 i.v. LD₅₀, or 7.14 mg venom neutralized per mL of antivenom. The EDs for the neutralization of procoagulant activities against bovine fibrinogen and human citrated plasma are 133.9 μL/mg and 104.0 μL/mg venom, correspondingly; whereas the ED₅₀s for the neutralization of hemorrhagic and necrotic activities are 122.8 μL/mg venom and 29.7 μL/mg venom, respectively (Tables 1 and 2). It is therefore not surprising to find that the Hemato polyvalent antivenom is also very effective in the neutralization of the lethality, procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom (Tables 1 and 2). Against 5 LD₅₀ (i.v.), the ED₅₀ of the polyvalent antivenom was determined to be 41.53 μL, which is equivalent to neutralization of 1.52 mg of *H. hypnale* venom per mL of the reconstituted Hemato polyvalent antivenom. In comparison, 1 mL of monovalent MPV antivenom neutralized only 0.89 mg of the venom. Comparison of the ED/ED₅₀s of neutralization of procoagulant, hemorrhagic and necrotic activities of the venom also showed that the Hemato polyvalent antivenom is far more effective ($p < 0.05$) than the monovalent MPV in neutralizing the toxic activities of the *H. hypnale* venom. In fact, the ED₅₀s of the neutralization of the procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom by the Hemato polyvalent antivenom is comparable to the ED/ED₅₀s against *C. rhodostoma* venom (Tables 1 and 2), though the antivenom is more effective against *C. rhodostoma* than *H. hypnale* in terms of neutralization of lethality (7.14 mg venom/mL antivenom, versus 1.52 mg venom/mL antivenom, $p < 0.05$).

The greater efficacy of the Hemato polyvalent antivenom than the monovalent MPV antivenom in the neutralization of *H. hypnale* venom suggested that the other two venoms (of *D. siamensis* and *C. albolabris*) used in the immunization scheme of prepara-

Table 2
Neutralization of the procoagulant, hemorrhagic and necrotic activities of *H. hypnale* and *C. rhodostoma* venoms by the monovalent MPV antivenom and Hemato polyvalent antivenom.

Toxic activity	Minimum dose	Neutralization by MPV antivenom (ED or ED ₅₀)	Neutralization by HP antivenom (ED or ED ₅₀)
Procoagulant	MCD		
<i>H. hypnale</i>	56.2 ± 1.3 μg/mL ^a	ED = 4.8 ± 0.1 μL ^a (432.1 ± 10.9 μL/mg venom)	ED = 1.3 ± 0.0 μL ^a (114.2 ± 2.7 μL/mg venom)
	55.1 ± 1.4 μg/mL ^b	ED = 4.2 ± 0.1 μL ^b (384.4 ± 11.2 μL/mg venom)	ED = 1.3 ± 0.0 μL ^b (121.3 ± 0.7 μL/mg venom)
<i>C. rhodostoma</i>	27.3 ± 0.3 μg/mL ^a	ED = 1.1 ± 0.0 μL ^a (209.2 ± 0.2 μL/mg venom)	ED = 0.7 ± 0.0 μL ^a (133.9 ± 0.3 μL/mg venom)
	24.9 ± 0.4 μg/mL ^b	ED = 0.8 ± 0.0 μL ^b (152.7 ± 3.7 μL/mg venom)	ED = 0.5 ± 0.0 μL ^b (104.0 ± 1.2 μL/mg venom)
Hemorrhagic	MHD		
<i>H. hypnale</i>	10.5 ± 0.6 μg	ED ₅₀ = 9.9 ± 0.3 μL (472.3 ± 13.9 μL/mg venom)	ED ₅₀ = 1.4 ± 0.1 μL (67.4 ± 5.1 μL/mg venom)
<i>C. rhodostoma</i>	24.0 ± 0.9 μg	ED ₅₀ = 7.3 ± 1.4 μL (151.7 ± 29.3 μL/mg venom)	ED ₅₀ = 5.9 ± 0.2 μL (122.8 ± 3.9 μL/mg venom)
Necrotic	MND		
<i>H. hypnale</i>	39.3 ± 1.6 μg	ED ₅₀ = 61.2 ± 1.7 μL (612.6 ± 17.5 μL/mg venom)	ED ₅₀ = 5.3 ± 0.1 μL (53.8 ± 0.59 μL/mg venom)
<i>C. rhodostoma</i>	28.7 ± 2.6 μg	ED ₅₀ = 2.7 ± 0.1 (38.1 ± 1.9 μL/mg venom)	ED ₅₀ = 2.1 ± 0.0 μL (29.7 ± 0.4 μL/mg venom)

Neutralization of hemorrhagic and procoagulant activities were determined by challenge with 2 MHDs and incubation with 2 MCDs, respectively, of the venom. For neutralization of necrotic activity, mice were challenged 2.5 MNDs of venom intradermally. Values are expressed as mean ± S.E.M. ($n = 3$ for procoagulant, hemorrhagic and necrotic activities).

^a Procoagulant activity tested on bovine fibrinogen.

^b Procoagulant activity tested on human citrated plasma.

tion of the polyvalent antivenom may contain venom toxins that are immunologically similar to some *H. hypnale* venom toxins. Our preclinical studies therefore suggest that both the monovalent MPV and the Hemato polyvalent antivenom may be useful in the antivenom treatment of systemic *H. hypnale* envenoming. The polyvalent antivenom might be the preferred one because of its greater efficacy, though the drawback is its higher cost (USD 60 per vial compared to USD 20 per vial for the monovalent antivenom). In view of the relatively low yield (average of 13 mg per milking) of venom from *H. hypnale* because of its relatively small size (Tan et al., 2010), and the strong neutralization capacity of the Hemato polyvalent antivenom, 1–2 vial (10–20 mL reconstituted antivenom) of the polyvalent antivenom would probably be sufficient in the treatment of most systemic *H. hypnale* envenomation. Nevertheless, while the results of the preclinical studies are promising, these studies must be followed by a randomized controlled clinical trial in the relevant regions, which is the final criterion for assessing the clinical efficacy and safety of the antivenom.

3.3. *In vitro* neutralization of the lethality of *D. russelii* and *E. carinatus sochureki* venom by the Hemato polyvalent antivenom

The ability of the Hemato polyvalent antivenom to neutralize the other two common vipers (*D. russelii* and *E. carinatus sochureki*) in Sri Lanka and India subcontinent was also investigated, as these two vipers are also common causes of snake bites in the region, and it is not always possible to identify the biting species in these snake bites. Our results showed that the Hemato polyvalent antivenom could neutralize the Sri Lankan *D. russelii* venom effectively, with an ED₅₀ of 7.52 µL/5 LD₅₀ (i.v.), or equivalent to 2.50 mg venom/mL antivenom. This is not surprising as the Hemato polyvalent antivenom was raised against a mixture of 3 venoms, including the Thai *D. siamensis* venom, which presumably contains some common antigens with the Sri Lanka *D. russelii* venom. The polyvalent antivenom, however, was not effective in the neutralization of *E. carinatus sochureki* venom: all 4 mice that were injected with 5 LD₅₀ (i.v.) died despite receiving a maximum of 200 µL of the antivenom. This result is not surprising as the toxinology of *E. carinatus sochureki* venom is very different from the three venoms used in the production of the Hemato polyvalent antivenom. Nevertheless, the ability of the Hemato polyvalent antivenom to neutralize *D. russelii* venom increases the potential benefit of the use of Hemato polyvalent antivenom in management of viper/pit viper bites in Sri Lanka and Southern India, as *D. russelii* is the second commonest cause of snake bite in the region, next to *H. hypnale* (Ariaratnam et al., 2009). Since clinically it is not always possible to distinguish between envenomation by *H. hypnale*, *D. russelii* and *E. carinatus*, double-sandwich ELISA should be developed to assist in biting species identification in future clinical trials of the antivenoms.

3.4. *In vivo* neutralization of *H. hypnale* venom using a rodent model of envenoming

To further evaluate the potential of the application of the Hemato polyvalent antivenom in the treatment of *H. hypnale* bites, we also carried out an *in vivo* neutralization experiment, using a rodent model. In this experiment, four mice received intravenous injection of 200 µL of Hemato polyvalent antivenom 5 min after i.m. injection of minimum lethal dose (400 µg per mouse) of *H. hypnale* venom. All four mice survived, and observations of the treated animals indicated that in addition to neutralization of the venom lethality of the venom *in vivo*, the antivenom also prevented or largely reduced the occurrence of venom-induced local tissue damage (myonecrosis and bleeding) of the venom. This observation is consistent with the result of *in vitro* neutralization of the necrotic

and hemorrhagic effects of *H. hypnale* venom by the polyvalent antivenom (Table 2).

Ariaratnam et al. (2008) reported in the series of humped-nose pit viper bites, 117 (39%) were systematically envenomed, all with hemostatic abnormalities and 30 with acute renal failure. We at this moment have not been able to assess the ability of the antivenoms to neutralize the nephrotoxicity due to the lack of sufficient amount of *H. hypnale* venom to develop a reliable *in vivo* nephrotoxicity assay. Nevertheless, coupled with our *in vitro* findings, the current *in vivo* results demonstrate further the potential benefits of the polyvalent antivenom in the treatment of *H. hypnale* bite.

4. Conclusions

Our results showed that both the monovalent MPV antivenom and the Hemato polyvalent antivenoms are effective in the neutralizations of the lethality and major toxic activities induced by *H. hypnale* venom when using *in vitro* and *in vivo* rodent assay protocols, in spite of the fact that the *H. hypnale* venom is not included in the immunizing mixture used in the production of the antivenom. Hence, this indicates that there are substantial immunological cross-reactivities between toxic components present in *H. hypnale* venom and the viperid venoms used in the production of the Hemato polyvalent antivenom. Extensive cross-neutralization between several *Bothrops* venoms and antivenoms has been described (Rojas et al., 2005; Bogarin et al., 2000). Our results therefore support the hypothesis that some antivenoms can be effective against heterologous venoms in other countries and may be helpful in situations where locally produced antivenoms are not available, as demonstrated by Otero et al. (1996).

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Enzymatic and toxinological activities of *Hypnale hypnale* (hump-nosed pit viper) venom and its fractionation by ion exchange high performance liquid chromatography

Tan CH (1, 2), Sim SM (1, 2), Gnanathasan CA (3), Fung SY (2, 4), Ponnudurai G (3), Pailoor J (5), Tan NH (4)

(1) Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; (2) The Center for Natural Products and Drug Research (CENAR), University of Malaya, Kuala Lumpur, Malaysia; (3) Division of Human Biology, International Medical University, Kuala Lumpur, Malaysia; (4) Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; (5) Department of Pathology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

Abstract: *Hypnale hypnale* (hump-nosed pit viper) has been recently identified as one of the medically important venomous snakes in Sri Lanka and on the southwestern coast of India. The characterization of its venom is essential for understanding the pathophysiology of envenomation and for optimizing its management. In the present study, the biological properties of *Hypnale hypnale* venom and venom fractions obtained using Resource Q ion exchange chromatography were determined. The venom exhibited toxic activities typical of pit viper venom, comparable to that of its sister taxon, the Malayan pit viper (*Calloselasma rhodostoma*). Particularly noteworthy were its high activities of thrombin-like enzyme, proteases, phospholipase A₂, L-amino acid oxidase and hyaluronidase. The thrombin-like enzyme was mainly acidic and distributed over several chromatography fractions, indicating its existence in multiple isoforms. The hemorrhagic and necrotic activities of the venom were likely associated with the proteolytic enzyme found mainly in the basic fraction. Phospholipase A₂ and phosphomonoesterase exist in both acidic and basic isoforms, while L-amino acid oxidase and hyaluronidase are highly acidic. The venom clotting activity on fibrinogens showed distinct species specificity in the following increasing order for clotting time: bovine < rabbit < goat < human < horse < dog, and was comparable to that of *C. rhodostoma* venom. Its clot formation on human fibrinogen is gradual and prolonged, a phenomenon suggestive of consumptive coagulopathy as a complication observed clinically. At an intramuscular sublethal dose, the venom did not cause acute kidney injury in a rodent model, contrary to the positive control group treated with *Daboia russelii* venom. Nephrotoxicity may result from higher venom doses in the context of coagulopathy, as a complication provoked by venom hematoxicity.

Key words: *Hypnale hypnale*, venom, enzymes, toxins, fibrinogen, nephrotoxicity.

INTRODUCTION

Snakes from the Viperidae family, including both vipers (viperines) and pit vipers (crotalids), are known to produce venoms that are principally hematoxic (1, 2). These venoms generally contain well over one hundred protein constituents, of which toxins belong to only a few major protein families that include such enzymes as serine proteases, Zn²⁺-metalloproteases, L-amino acid oxidase and group II PLA₂; as well as non-enzymatic proteins (constituting a smaller

portion) including disintegrins, C-type lectins, myotoxins, cysteine-rich secretory protein (CRISP) toxins etc. (3, 4). This context reflects the emergence of toxins from a restricted set of physiological protein families recruited into the venom proteome at the base of the Colubroidea radiation, ideal for their primary role in predation and defense (5, 6). Nevertheless, envenomation of humans causes serious public health problems, for which it has been duly categorized as a “disease” according to the WHO International Classification of Disease (ICD-10),

and appropriately categorized as a Neglected Tropical Disease (7). This problem also affects most tropical and subtropical countries, many of which are still underdeveloped, hence its apt description as a disease of poverty (8). Despite this, the envenomation issue remains systemically neglected in many parts of the world (9).

In South Asia, annual mortality due to snakebites has been estimated to be 1,000 in Sri Lanka and 20,000 in India (10). *Hypnale hypnale* (hump-nosed pit viper) has been recently recognized as a medically important venomous snake in Sri Lanka and on the southwestern coast of India (11, 12). It is now listed as a category I snake of medical importance by the World Health Organization, owing to its wide distribution and high prevalence of bites which cause significant morbidity and mortality in the region (13). This snake is mainly nocturnal and both arboreal and terrestrial. It inhabits both wet and dry deciduous zones; can be found in settings ranging from secondary forests, hilly areas, various plantations to low land gardens and even latrines within home compounds. Being well camouflaged and tiny in size (body length rarely exceeds 0.5 m), it is easily missed and trodden upon even in the daytime, resulting in its defensive bite (11). In Sri Lanka, it is not uncommon to come across *H. hypnale* envenoming even in the capital city of Colombo, although more cases occur in the suburban and rural areas.

Although the local effects of its bites were well observed and documented, it remains controversial after more than a century as to whether *H. hypnale* bites could be fatal (14-18). Recent clinical studies indicated that envenomation by this snake resulted in local edema, necrosis, regional lymphadenopathy, as well as systemic effects characterized by hemostatic dysfunction (coagulopathy, fibrinolysis, thrombocytopenia, spontaneous systemic hemorrhage) and acute renal failure, with an overall fatality rate of 1.7% in authenticated cases (11, 12, 19-22). To date, apart from the *Calloselasma rhodostoma* antivenoms (produced by the Thai Red Cross Society) which were shown to confer paraspecific neutralization in rodent models, there is no specific antivenom that is clinically effective against the *H. hypnale* venom (11, 12, 23).

In order to address the envenoming issue holistically, the venom's toxinological profile should be studied to elucidate its pathogenesis. In

this paper, we report the biological properties of the venom and its ion exchange chromatography-fractionated components, using *in vitro* and/or *in vivo* methods. Its clotting activity on several mammalian fibrinogens was also investigated. A rodent model was also used to study the *in vivo* nephrotoxic effects of this venom.

MATERIALS

Venoms

Hypnale hypnale venom was a pooled sample obtained from the milking of adult snakes captured in Sri Lanka (Gamapha, Kelaniya, Avissawela, Colombo regions). The snakes were kept at the snake farm in the University of Colombo, Sri Lanka and were identified by Anslem de Silva, an expert herpetologist. Venoms of *Calloselasma rhodostoma*, *Cryptelytrops purpureomaculatus* and *Daboia russelii* (Myanmar) were purchased from Latoxan (Valence, France).

Reagents and Animals

All reagents and chemicals were purchased from Sigma Chemical Company (USA) and were of analytical grade. Albino mice (ICR strain) and Sprague Dawley rats were supplied by the Laboratory Animal Center, Faculty of Medicine, University of Malaya. The use of animals was approved by the institute and these animals were handled according to the guidelines of the Council for International Organizations of Medical Sciences (CIOMS) on animal experimentation (24).

METHODS

Fractionation of *H. hypnale* venom by Resource® Q Ion-Exchange Chromatography

Five milligrams of the venom dissolved in 200 µL of starting buffer (20 mM Tris-HCl, pH 8.5) was filtered through a 0.2 µm pore size syringe filter. The filtrate was then injected into Resource® Q ion-exchange column (GE Healthcare, USA) (6.4 mm x 30 mm, 1 mL gel volume, 15 µm particle size, 200-10,000 Å) pre-equilibrated with starting buffer, and subsequently eluted by a linear, 0.0 to 0.5 M sodium chloride gradient (0-30% from 5 to 30 minutes, followed by 30-100% from 30 to 55 minutes), at the flow rate of 1 mLmin⁻¹. The solvent delivery and gradient formation over 60 minutes was achieved by using the Shimadzu LC-20AD® high performance liquid chromatography

(HPLC) system (Japan). Protein peaks were monitored by measuring the absorbance at 280 nm. The protein content of the fractions was determined by the Bradford method (25).

Determination of Enzymatic Activities

This portion of the study was done on the venoms of *H. hypnale* and *Calloselasma rhodostoma* for comparison, as well as on the HPLC-isolated protein fractions of *H. hypnale* venom. Two independent experiments were carried out on each test for each sample, and the average results were reported.

Protease assay

Protease activity was measured by modification of the method described by Kunitz (26). One milliliter of 1% casein in 0.25 M sodium phosphate buffer, pH 7.75, and 50 μ L of sample solution were incubated for 30 minutes at 37°C. The reaction was terminated by adding 1 mL of 5% trichloroacetic acid. After centrifugation at 10,000 \times g for ten minutes, the absorbance of the supernatant was measured at 280 nm. One unit of protease activity was defined as an increase of one absorbance unit per hour at 280 nm.

Phospholipase A₂ assay

Phospholipase A₂ activity was determined by the acidimetric method (27). The egg yolk substrate suspension was prepared by mixing one part chicken egg yolk, one part 18 mM calcium (II) chloride, and one part 8.1 mM sodium deoxycholate. The pH of the substrate suspension was adjusted to 8.0 with 1 M sodium hydroxide. The suspension was stirred to ensure good mixing. One hundred microliters of venom solution was added to 15 mL of the substrate suspension and the rate of decrease in pH was recorded using a pH meter. A decrease of 1 pH unit of the egg yolk suspension corresponded to 133 μ mol of fatty acids released.

Arginine ester hydrolase assay

Arginine ester hydrolase was assayed using α -benzoyl arginine ethyl ester as substrate (28). The assay mixture contained 0.95 mL of 0.8 mM substrate in 0.05 M Tris-HCl buffer, pH 7.8, and 50 μ L of sample solution. The reaction was monitored by measuring the rate of increase in absorbance at 255 nm. The difference in the extinction coefficient at 255 nm is 815 $\text{cm}^{-1}\text{M}^{-1}$.

Phosphodiesterase assay

Phosphodiesterase activity was determined by a method modified from Lo *et al.* (29). One hundred microliters of sample was added to an assay mixture containing 0.5 mL of 2.5 mM calcium bis-p-nitrophenylphosphate, 0.3 mL of 0.01 M magnesium sulfate and 0.5 mL of 0.17 M veronal buffer, pH 9.0. The hydrolysis of the substrate was monitored by measuring the rate of increase of absorbance at 400 nm. The extinction coefficient is 8100 $\text{cm}^{-1}\text{M}^{-1}$.

Alkaline phosphomonoesterase assay

Alkaline phosphomonoesterase activity was determined by a method modified from Lo *et al.* (29). One hundred microliters of sample was added to an assay mixture containing 0.5 mL of 0.5 M glycine buffer (pH 8.5), 0.5 mL of 10 mM p-nitrophenylphosphate and 0.3 mL of 0.01 M magnesium sulfate. The mixture was incubated at 37°C for 30 minutes. At the end of the incubation period, 2 mL of 0.2 M sodium hydroxide was added and allowed to stand for 20 minutes at room temperature. The absorbance at 400 nm was then measured. The extinction coefficient is 18500 $\text{cm}^{-1}\text{M}^{-1}$.

5'-Nucleotidase assay

5'-Nucleotidase activity was determined using 5'-AMP as substrate (30). One hundred microliters of sample was added to an assay mixture containing 0.5 mL of 0.02 M 5'-AMP (preadjusted to pH 8.5), 0.5 mL of 0.2 M glycine buffer, pH 8.5, and 0.1 mL of 0.1 M magnesium sulfate. The mixture was incubated for ten minutes at 37°C, and the reaction was terminated by the addition of 1.5 mL of 10% trichloroacetic acid. The ascorbic acid method was used to determine the inorganic phosphate content (31). To the above mixture, 1 mL of ascorbic acid reagent – containing equal parts of 3 M sulfuric acid, 2.5% ammonium molybdate, 10% ascorbic acid and water – was added. The mixture was left at room temperature for 30 minutes, and the absorbance at 820 nm was then measured. A standard curve was constructed using known concentrations of inorganic phosphate.

Hyaluronidase assay

Hyaluronidase activity was determined turbidimetrically (32). The assay mixture contained 0.45 mL of 0.2 M acetate buffer, pH 5.0,

containing 0.15 M sodium chloride and 0.2 mg of human umbilical cord hyaluronic acid and 50 μ L of sample solution. The mixture was incubated for 1 h at 37°C. The reaction was terminated by the addition of 1 mL of 2.5% cetyltrimethylammonium bromide in 2% sodium hydroxide solution. After 30 minutes, the absorbance at 400 nm was measured. Enzymatic activity was expressed as NFU/mg (National Formulary Units). Appropriate dilutions of sample were performed to ensure that the difference in absorbance between the blank and sample was less than 0.4. Hyaluronidase (EC 3.2.1.35), originating from bovine testes, was purchased from Sigma Chemical Co. (USA) and used as a standard; its activity is 270 NFU per mg. Several dilutions of hyaluronidase representing a series of NFUs were tested on solution containing 0.2 mg of hyaluronic acid. The absorbance values were plotted against the NFUs for calibration.

L-amino acid oxidase

L-amino acid oxidase activity was determined as described by Tan *et al.* (33). Fifty microliters of horseradish peroxidase (100 purpurogalin unit/mg) was added to 0.9 mL of 0.2 M triethanolamine buffer, pH 7.6, containing 0.1% L-leucine and 0.0075% o-dianidisine and incubated for three minutes at room temperature. Sample solution (50 μ L) was then added and the increase in absorbance at 436 nm was measured. The molar absorption coefficient is $8.31 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$.

Determination of Lethality, Hemorrhagic, Procoagulant and Necrotic Activities

Median lethal dose (LD₅₀)

The LD₅₀ values of *H. hypnale* venom were determined by intraperitoneal injection of the venom into mice (18–22 g, n = 4 per dose) whose survival ratio was recorded after 48 hours. The intraperitoneal LD₅₀ (and the 95% confidence intervals, CI) of the venom was calculated using the Probit analysis method of Finney (34). The intramuscular LD₅₀ value of *H. hypnale* was based on a previous determination from the same laboratory as reported by Tan *et al.* (23), while that for *D. russelii* was determined via intramuscular injection of the venom into hind legs of mice (n = 4 per dose), and analyzed as previously mentioned.

Hemorrhagic activity

Hemorrhagic activity was determined according to Theakston and Reid (35) with modifications. The venom dissolved in phosphate-buffered saline (50 μ g in 50 μ L) or the venom fraction (50 μ L) was injected intradermally into the shaved dorsal skin of lightly anesthetized mice. After 90 minutes, the animals were euthanized with an overdose of diethyl ether. The dorsal skin was removed carefully, and the mean diameter of the hemorrhagic lesion on the inner surface of the skin was measured.

Procoagulant activity

Procoagulant activity was determined by the method described by Bogarin *et al.* (36). The venom (50 μ g in 0.1 mL) or venom fraction (0.1 mL) was added to 0.2 mL of bovine fibrinogen solution (2 g/L) at 37°C. The coagulation time (time required for fibrin strands to form) was recorded. A shorter coagulation time suggests higher procoagulant activity.

Necrotic activity

Necrotic activity was determined by a modified version of the method of Theakston and Reid (35). The venom, (50 μ g in 50 μ L) or venom fraction (50 μ L) was injected intradermally into the shaved dorsal skin of lightly anesthetized mice. After 72 hours, the animals were euthanized with an overdose of diethyl ether, the dorsal skin was removed, and the mean diameter of the necrotic lesion was measured.

Fibrinogen clotting activity

The fibrinogen clotting activity (procoagulant effect) of *H. hypnale* venom was tested further on fibrinogens from several mammalian sources. Venoms from two other Asiatic pit vipers, *C. rhodostoma* and *C. purpureomaculatus*, well known for their procoagulant effects, were studied simultaneously for comparison (37, 38).

The venom (50 μ g in 0.1 mL) was added to 0.2 mL of fibrinogen solution from various animal sources (2 g/L) at 37°C. The coagulation time (time elapsed for fibrin strands to form) was recorded. To study the time course of venomous procoagulant activities, each venom (50 μ g in 0.1 mL) was added to 0.2 mL of fibrinogen solution (2 g/L) preincubated at 37°C. The absorbance of the mixture at 450 nm was read for ten minutes to monitor the formation and degradation of fibrin clots.

Nephrotoxicity study

Sprague dawley rats were divided into three groups of three animals each: normal saline (NS) group, *H. hypnale* venom (HV) group and *Daboia russelii* venom (DV) group. The NS group and DV groups represented, respectively, the negative and positive controls. Rats from each group received intramuscular injections of treatment as follows: 200 µL of normal saline, or 200 µL of venom solution corresponding to one third of the venom's intramuscular LD₅₀. The rats were kept for 24 hours in metabolic cages with access to feed and water *ad libitum*. Urine was collected throughout 24 hours, while blood was collected via cardiac puncture under anesthesia at the end of experiment. The urine and blood were sent for biochemical analysis, to an outsourced service at a pathology analytical laboratory. Major organs harvested from rats upon death following cardiac punctures were sent for histopathological studies. Specimens were fixed in 10% formalin, followed by paraffin wax-embedding, sectioning of tissue, dewaxing, rehydration, and staining with hematoxylin and eosin. The sections were cover-slipped and images were captured using a Nikon Research Trinocular Teaching Microscope Eclipse 80i® (Japan) equipped with a camera.

Statistical analysis

The variability of lethality assays was expressed within 95% confidence intervals (CI). The significance of the differences of the means was determined by two-tailed unpaired Student's *t*-test or one-way ANOVA. Statistical analyses were carried out using the statistical software SPSS.

RESULTS AND DISCUSSION

Milking of Snake Venom

In an investigation of venom yield, the milking of six adult *H. hypnale* snakes yielded 10.0 mg, 8.7 mg, 11.4 mg, 20.0 mg, 12.2 mg and 15.6 mg, respectively, of dried venom. The average amount of venom obtained per milking is therefore 13 mg (± 4.2 mg). Hence the maximum amount of venom injected per bite is expected to be less than 20 mg, much lower than the roughly 50 mg per yield reported in its sister taxon, *C. rhodostoma* (37). This is predictable as the body size of *H. hypnale* is small and rarely grows beyond 0.5 m.

Lethality of the Venom

H. hypnale venom has an intraperitoneal median lethal dose (LD₅₀) of 6.0 µg/g (95% CI 4.2-8.1 µg/g) of mouse body weight, which is intermediate between the intravenous LD₅₀ (0.9 µg/g) and the intramuscular LD₅₀ (13.7 µg/g) reported by our research group in an earlier study (23). This suggests that the potency of toxins causing lethality differs depending on the administration route. A much lower intravenous LD₅₀ indicates that the venom is likely to be principally hematoxic in action, while the intraperitoneal LD₅₀ is higher than the intravenous value possibly due to delayed an/or incomplete absorption (as in first pass phenomenon) of the toxins before reaching the systemic circulation (39, 40). The fact that the intraperitoneal LD₅₀ value is lower than that of the intramuscular route is likely due to higher bioavailability given that more venom is quickly absorbed intraperitoneally (larger surface area and richer blood supplies) without having to bind and interact with muscle and connective tissues.

The median intramuscular lethal dose of *D. russelii* venom tested in mice was 0.70 µg/g (95% CI, 0.45-0.9 µg/g) of body weight, which was determined for use in the nephrotoxicity study.

Biological Properties of *H. hypnale* and *C. rhodostoma* Venoms

The *H. hypnale* venom used in this study showed higher activities of proteases, phospholipase A₂, L-amino acid oxidase and hyaluronidase than those found in a previous report (41) (Table 1). This may be due to ontogenic differences as the venom used in this study was a recently pooled sample. The procoagulant activity, however, did not differ markedly between the two reports.

H. hypnale venom exhibited enzymatic properties similar to *C. rhodostoma* venom (Table 1), which are quite typical of pit viper venoms, notably the presence of arginine ester hydrolase and potent protease activities. Particularly noteworthy is the substantially higher phospholipase A₂ activity in *H. hypnale* venom; in comparison, *C. rhodostoma* venom usually exhibits rather / very low phospholipase A₂ activity. Phospholipases A₂ are known to exhibit multiple pharmacological activities, including lipid membrane damage, myotoxicity and myonecrosis (42, 43). High amounts of phospholipase A₂, proteases and L-amino acid

oxidase, known for their cytotoxic and tissue-damaging properties, are likely responsible for the local envenoming features, e.g. edema, necrosis and hemorrhagic blistering commonly seen after *H. hypnale* bites (11, 12, 43-45). In addition, hyaluronidase that presents substantially in the venom can synergistically potentiate the venom propagation and its local effects, contributing to severe local tissue destruction (46).

H. hypnale venom also showed potent procoagulant or thrombin-like enzyme activities (lower than that of *C. rhodostoma*), as well as hemorrhagic and necrotic properties (higher than or similar to that of *C. rhodostoma*). These activities are hallmark of the pathophysiology induced by pit viper venoms. The general similarities in the biological activities of *H. hypnale* venom and *C. rhodostoma* venom are expected as they are closely related phylogenetically, hence the possibility of sharing similar venom antigens (47, 48).

Fractionation of *H. hypnale* venom by Resource® Q Anion Exchange Chromatography

Ion exchange chromatography separates proteins based on their ionic charges, and has been useful in snake venom fractionations as it does not affect the biological activity of eluted proteins, hence allowing their characterizations (33, 38). With Resource® Q column, an anion exchanger, *H. hypnale* venom was fractionated yielding twelve major fractions (Figure 1). Protein estimation by

the Bradford method (25) showed that fraction 12 has very little protein content. Fractions 1 and 2 are unbound fractions and contain basic proteins, while the remaining fractions generally contain proteins of an increasingly acidic nature.

Biochemical Properties of the Protein Fractions of *H. hypnale* venom

Table 2 shows the biological properties of the 12 fractions obtained from Resource® Q chromatography of *H. hypnale* venom. Fraction 1, containing the basic proteins, was certainly heterogeneous, as it possessed multiple enzyme activities including proteases, phospholipase A₂, alkaline phosphomonoesterase, phosphodiesterase and 5'-nucleotidase activities. It was also the only fraction that exhibited hemorrhagic and necrotic activities, and both of these toxic effects were likely associated with the proteolytic activity, as reported in viperid metalloproteinases (1). Both fractions 1 and 3 exhibited phospholipase A₂ activity, suggesting that the venom contains at least two forms of phospholipase A₂. This finding is in accord with a previous report (49) where only two out of four PLA₂ isoforms isolated from *H. hypnale* venom, using combined gel filtration and reversed-phase chromatography, exhibited enzymatic activities. We suggest that these two reported enzymes belong to a basic form (fraction 1) and an acidic form (fraction 3), respectively.

Hydrolytic enzymes – such as phosphodiesterase (a nuclease), 5'-nucleotidase

Table 1. Enzymatic, hemorrhagic and necrotic activities of *H. hypnale* and *C. rhodostoma* venoms

Activity Species	PRO	PLA ₂	LAO	AEH	PME	PDE	5'-Nuc	HYA	TLE	HEM	NEC
<i>H. hypnale</i> [#]	15.8	191.5	191.7	10.05	4.8	4.1	1.8	115.5	20	23	7.5
<i>C. rhodostoma</i> [#]	18.5	79.8	165.4	8.96	4.6	18.5	1.9	76.6	10	16	8.0
<i>H. hypnale</i> [@]	7.8	126	110	15.2	12	6	2.7	85	22	1.6*	ND

PRO: protease, unit/mg; PLA₂: phospholipase A₂, μmol/min/mg; LAO: L-amino acid oxidase, nmol/min/mg; AEH: arginine ester hydrolase, μmol/min/mg; PME: alkaline phosphomonoesterase, nmol/min/mg; PDE: phosphodiesterase, nmol/min/mg; 5'-Nuc: 5'-nucleotidase, μmol/min/mg; HYA: hyaluronidase, NFU/mg; TLE: thrombin-like enzyme (procoagulant enzyme), s/50 μg venom; Hem: hemorrhagin, mm/50 μg venom; NEC: necrosis, mm/50 μg venom; ND: not determined. Values were the average results from two independent experiments.

[#] Present study; [@] reference to Tan and Ponnudurai (41); * the unit was expressed as the product of two perpendicular diameters of the lesion, which is different from the unit defined in the present study.

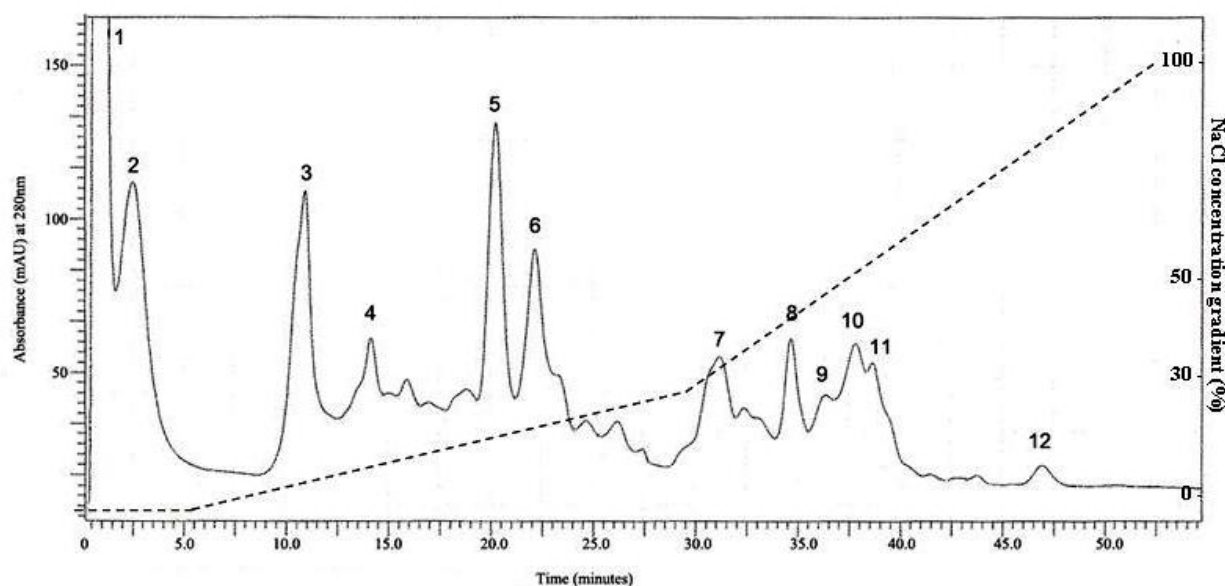


Figure 1. Fractionation of *Hypnale hypnale* venom with Resource® Q anion exchange chromatography. The venom (5 mg) was injected into the column equilibrated with 20 mM Tris-HCl, pH 8.5, and eluted by a linear, 0.0 to 0.5 M NaCl gradient (0-30% from 5 to 30 minutes, followed by 30-100% from 30 to 55 minutes). Flow rate was 1 mL/min. (----): NaCl concentration gradient (%)

and alkaline phosphomonoesterase – are high molecular weight proteins known to be widely distributed across many snake taxa, but their toxic effects have been less extensively characterized partly due to the fact that these enzymes share similar substrates and biochemical properties (50). In general, our results showed that all three enzymes were found in *H. hypnale* venom in appreciable amounts, particularly phosphodiesterase, which is usually abundant in crotalid venoms. Phosphodiesterase had been associated with a drop in mean arterial pressure and locomotor depression, presumably due to reduced cAMP levels (51). This feature, however, has not been reported in systemic envenomation by *H. hypnale*, implying that its function might be more digestive than purely toxinological. The chromatography result also showed that 5'-nucleotidase and phosphomonoesterase from *H. hypnale* venom exist in both basic and acidic isoforms. It is intriguing to note the existence of acidic phosphomonoesterase in this venom, as this enzyme, contrary to the basic/alkaline phosphomonoesterase, has so far only been purified from sea snake venoms and rarely been reported in other snake venoms (52).

Thrombin-like activity was found to be widespread from fraction 2 to fraction 8,

indicating that the enzyme likely existed in multiple isoforms. The two fractions (fraction 5, 6) with the strongest thrombin-like activity were also the two fractions that hydrolyzed arginine esters, suggesting that the thrombin-like enzymes exhibited arginine ester hydrolase activity, a property similar to that of ICR-50 arvin, a coagulant enzyme derived from *C. rhodostoma* venom (28). The enzyme esterolytic activity towards *N*- α -benzoyl-L-arginine ethyl ester indicated its specific binding to the arginine residues in its substrate. The venom also contained one or more forms of L-amino acid oxidase which appeared more acidic than many other enzymes, and its activity was noted to be lost by freezing. Such freeze-labile activity had been reported and the mechanism is presumably due to a limited conformational change of the enzyme structure (53, 54). Fraction 12, which contained few proteins, was found to be devoid of enzymatic activities. As demonstrated, venom fractionation helps elucidate the different components in venoms allowing their toxic characterizations for pathophysiological correlations. Nonetheless, it also opens the door to manipulating specific toxins in search of potential therapeutic compounds, as already observed in drug discoveries of ancrod, captopril etc. (55).

Table 2. Enzymatic, hemorrhagic and necrotic activities of major protein fractions of *H. hypnale* venom obtained from Resource Q ion-exchange chromatography

Fraction \ Test	AMT	PRO	PLA ₂	LAO	AEH	PME	PDE	5'-Nuc	HYA	TLE	HEM	NEC
1	240	8.3	79.8	–	–	0.5	9.8	0.18	–	–	9	6
2	59	–	–	–	–	–	–	–	–	470	–	–
3	77	–	159.6	–	–	–	–	–	–	212	–	–
4	32	–	–	–	–	–	–	0.15	–	115	–	–
5	47	–	–	–	9.8	–	–	0.30	–	84	–	–
6	58	–	–	–	2.5	–	–	0.66	–	77	–	–
7	28	–	–	–	–	–	–	–	–	146	–	–
8	18	–	–	–	–	1.7	–	–	–	610	–	–
9	12	–	–	13.8	–	0.9	–	–	6.9	–	–	–
10	41	–	–	11.3	–	0.1	–	–	46.9	–	–	–
11	10	–	–	–	–	–	–	–	6.6	–	–	–
12	4	–	–	–	–	–	–	–	–	–	–	–

AMT: Total protein amount recovered in each respective fraction, µg; PRO: protease, unit/mL; PLA₂: phospholipase A₂, µmol/min/mL; LAO: L-amino acid oxidase, nmol/min/mL; AEH: arginine ester hydrolase, µmol/min/mL; PME: alkaline phosphomonoesterase, nmol/min/mL; PDE: phosphodiesterase, nmol/min/mL; 5'-Nuc: 5'-nucleotidase, µmol/min/mL; HYA: hyaluronidase, NFU/mL; TLE: thrombin-like enzyme (procoagulant enzyme), s/100 µL; HEM: hemorrhagic activity, mm/50 µL; NEC: necrotic activity, mm/50 µL, –: absent activity. Values were the average results from two independent experiments.

Fibrinogen Clotting Activity

The clotting activity of *H. hypnale* venom showed distinct species differences (Table 3). The clotting time of mammalian fibrinogens by *H. hypnale* venom was in the increasing order as follows: bovine < rabbit < goat < human < horse < < dog. The clotting activity of *C. rhodostoma* venom exhibited a similar pattern (most and least active towards the bovine and dog, respectively) indicating that the thrombin-like activity of the venoms of these two phylogenetically related crotalids exhibited similar specificity towards fibrinogens. On the other hand, the specificity of the clotting activity of *C. purpureomaculatus* venom (bovine < goat < human < horse < < rabbit, dog) was rather different, and generally showed a lower clotting potency for bovine, goat and human fibrinogens ($p < 0.05$).

The clotting activities of the three venoms were also monitored spectrophotometrically whereby the turbidity of the reaction mixture was continuously monitored for absorbance at 450

nm. The investigation of the time course of clot assembly had also been described by Pirkle *et al.* (56). Generally, the appearance of fibrin or clots would result in turbidity, while degradation of the clots would reduce this property. Figure 2 (A to D) shows that both *H. hypnale* and *C. rhodostoma* venoms caused rapid clotting with fibrinogens from bovine, goat, horse and rabbit specimens. Dissolution of clots was rapid in the case of goat (Figure 2 – B) and horse fibrinogens (Figure 2 – C) (turbidity reduced from 30 seconds). Both venoms were active in the clotting of human fibrinogen (Figure 2 – E), showing a slow but prolonged clotting effect. *C. purpureomaculatus* venom generally demonstrated moderate to low clotting activities, except for the case of dog fibrinogen (Figure 2– F) where it appeared more active than the venoms of *H. hypnale* and *C. rhodostoma*.

We suggest that a rapid clotting profile implies the possibility that the venom may precipitate acute thrombotic syndrome that can be instantly

Table 3. Procoagulant effects of venoms from three pit vipers on mammalian fibrinogens

Venom	<i>H. hypnale</i>	<i>C. rhodostoma</i>	<i>C. purpureomaculatus</i>
Source of fibrinogen	Clotting time* (s) (mean \pm SEM)	Clotting time (s) (mean \pm SEM)	Clotting time (s) (mean \pm SEM)
Rabbit	18.3 \pm 0.9	30.7 \pm 1.2	Turbidity > 120 s
Horse	42.2 \pm 1.0	40.1 \pm 0.6	Turbidity > 60 s
Dog	Turbidity** > 180 s	Turbidity > 180 s	Turbidity > 80 s
Goat	30.3 \pm 0.9	33.0 \pm 0.6	71.0 \pm 0.6
Bovine	14.3 \pm 0.3	11.3 \pm 0.9	56.7 \pm 0.7
Human	32.7 \pm 0.7	28.4 \pm 0.8	75.0 \pm 0.6

* Clotting time is defined as the time (s) for apparent fibrin strands to appear in the mixture of venom and fibrinogen. ** Turbidity refers to cloudiness appearing in the mixture of venom and fibrinogen, which may be interpreted as the formation of fibrin clots. Results are expressed as the mean \pm SEM from three experiments.

fatal, as observed in an experiment where a rabbit was injected with *H. hypnale* venom intravenously (unpublished data). In cases where clot formation is less rapid, or is accompanied by a concurrent degradation, the most likely cause of death would be a consumptive coagulopathy that gradually affects hemostasis, causing prolonged bleeding and a “slower” death. This is consistent with main clinical features reported in cases of envenomation by *C. rhodostoma* (57, 58) and *H. hypnale* (11, 12). Deranged hemostasis leading to systemic bleeding has been the main feature in systemic envenomation by these crotalids. This severe, potentially fatal complication might only manifest itself temporarily following the bite as the pre-existing coagulant factors get depleted or rendered inactive by the venom more rapidly than their production, hence tilting the hemostatic balance towards a tendency to bleed. A recent example was that of an extensive retroperitoneal hemorrhage only detected five weeks after a bite by *H. hypnale* in an adult patient (59).

Nephrotoxicity Study

Ariaratnam *et al.* (11) reported a series of 302 hump-nosed pit viper bites in which 117 (39%) were systematically envenomed, all with hemostatic abnormalities, while 30 presented with acute renal failure. The kidney, as a highly vascularized excretory organ, is certainly vulnerable to acute injury by venom toxicity. The injury mechanisms can be either by direct effect of the toxins or secondary to hemostatic defect, intravascular hemolysis,

shock, immune response or rhabdomyolysis. Hemotoxic and myotoxic venoms from viperids or crotalids are a rich source of enzymes that include phospholipases, endopeptidases, L-amino acid oxidases and metalloproteases which can directly cause cellular damage and kidney injury (60, 61). In the present study, *D. russellii* venom from Myanmar was administered to the positive control group (DV group) to produce acute kidney injury as the venom was known to be highly nephrotoxic (62-65).

Rats from both HV and DV groups treated with a sublethal dose (1/3 LD₅₀) of venoms of *H. hypnale* and *D. russellii* respectively, exhibited envenomation signs e.g. local hemorrhage, malaise, drowsiness and limping in the first few hours. All animals survived the 24-hour observation period. Gross inspection of urines showed marked hematuria among the DV group, with no inter-group difference in the urine volumes. Biochemical and microscopic urinalysis revealed significant proteinuria and hematuria in the DV group compared to the HV and NS groups ($p < 0.05$) (Table 4). The urea and creatinine levels, used as indicators for acute renal failure, were observably higher in the DV group although this elevation was insignificant in relation to the other two groups (Table 4), a finding probably attributable to the fact that the observation period was not sufficiently long. Nevertheless, the significant proteinuria and hematuria are sufficient indication that the injected *D. russellii* venom did cause acute nephrotoxicity in the animals. The absence of these features in rats

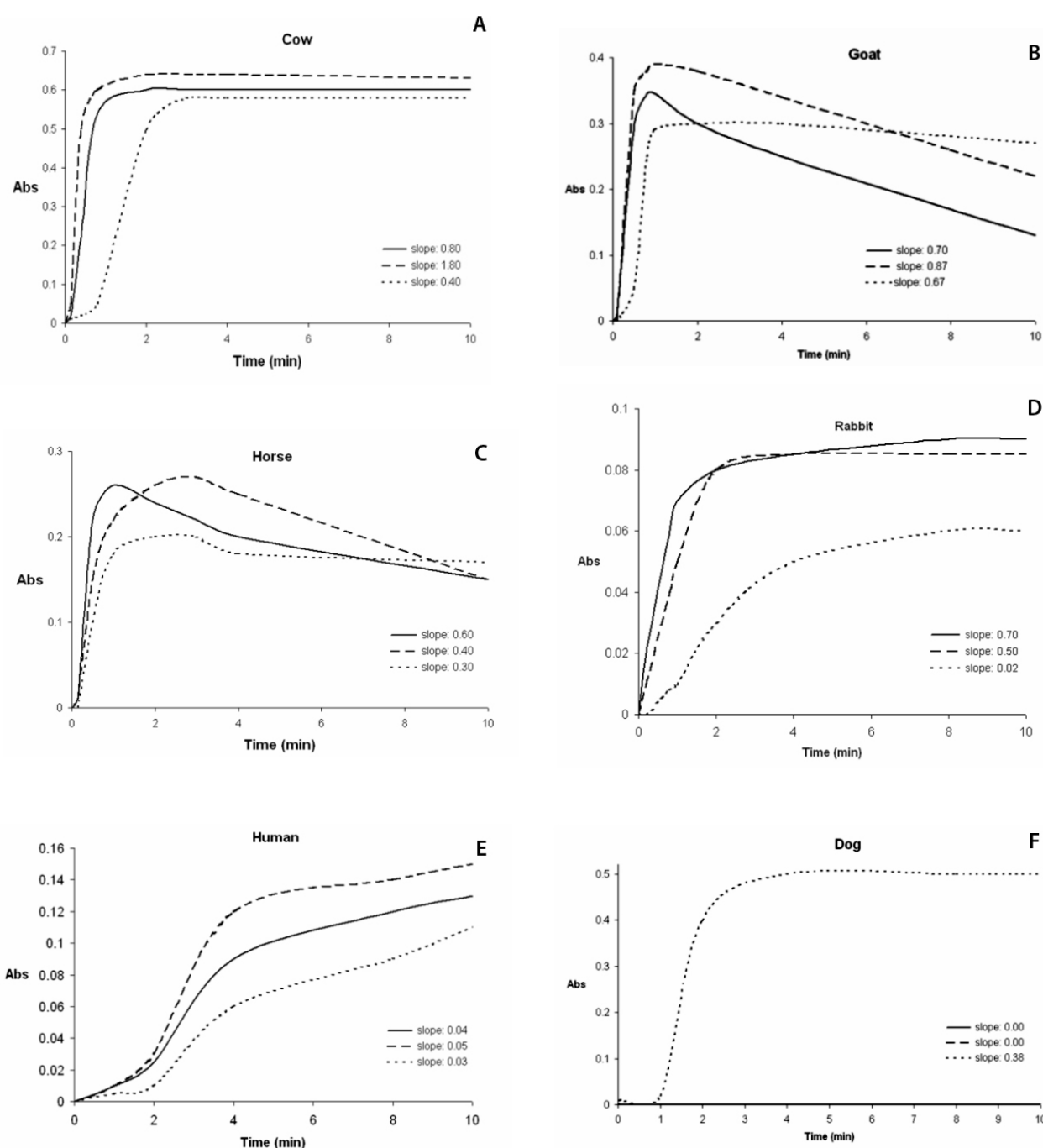


Figure 2. Time course of thrombin-like enzyme activity of three different crotalid venoms on fibrinogens from several mammals (A: cow; B: goat; C: horse; D: rabbit; E: human; F: dog). The slope (gradient) for each curve is calculated from the steepest upward portion of the graph representing the maximal clotting rate expressed as absorbance/minute. Absorbance reflects the change in mixture clarity, which is responsible for the formation of fibrin strands as well as their dissolution/degradation. "Clot" used in this discussion represents the formation of fibrins. The crotalid species are: *H. hypnale*; --- *C. rhodostoma*; *C. purpureomaculatus*.

injected with a similar sublethal dose of *H. hypnale* venom suggested that the given dose of the venom does not cause direct nephrotoxicity. This is further demonstrated by light microscopic examination of histological cuts from the kidney (data not shown) and other vital organs (heart,

lung, liver, spleen and diaphragm) of rats injected with the sublethal dose of *H. hypnale* venom, where all tissues appeared histologically intact. Thus, a tentative explanation of nephropathy observed in some of the victims (11, 66) is that the kidney damage is likely the result of complications from

Table 4. Effects of sublethal dose (1/3 intramuscular LD₅₀) of *H. hypnale* and *D. russelii* venoms on blood and urine parameters (n = 3)

Parameter (mean ± SEM)	Group of rat	Normal saline	<i>Hypnale hypnale</i> venom	<i>Daboia russelii</i> venom
Urine				
Color		Straw	Straw	Reddish
Appearance		Clear	Clear	Hazy
Volume (mL)		4.2 ± 0.7	4.0 ± 0.8	3.9 ± 0.7
Protein (+)		0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.5*
Blood (hematuria) (+)		0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.8*
Microscopic red blood cell		0.0 ± 0.0	0.0 ± 0.0	3.3 ± 0.9*
Nitrite (+)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Bacteria (+)		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Blood				
Urea (mmol/L)		6.0 ± 0.8	6.9 ± 0.6	14.5 ± 7.0
Creatinine (μmol/L)		18.7 ± 3.2	18.0 ± 3.6	32 ± 13.0

* Significantly different from the other two groups, p < 0.05, ANOVA

the venom's hematoxic effects mediated through its procoagulant and possibly fibrinolytic actions. This suggestion is in line with the view that the nephropathy was associated with disseminated intravascular coagulopathy (61).

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CONFLICTS OF INTEREST

There is no conflict.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Animal Care and Use Committee of the University of Malaya [ethics reference number: PM/03/03/2010/FSY(R)]. Moreover, animals were handled according to the guidelines given by CIOMS on animal experimentation.

CORRESPONDENCE TO

CHOO HOCK TAN, Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. Phone: +60 3 79674951. Fax: +60 3 79674791. Email: tanchoohock@gmail.com or tanch@um.edu.my.

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Immunological properties of *Hypnale hypnale* (hump-nosed pit viper) venom: Antibody production with diagnostic and therapeutic potentials

Choo Hock Tan^{a,*}, Nget Hong Tan^b, Si Mui Sim^a, Shin Yee Fung^b, Christeine Ariarane Gnanathan^c

^a Department of Pharmacology, Faculty of Medicine, and CENAR, University of Malaya, 50603 Kuala Lumpur, Malaysia

^b Department of Molecular Medicine, Faculty of Medicine, and CENAR, University of Malaya, Kuala Lumpur, Malaysia

^c Department of Clinical Medicine, Faculty of Medicine, University of Colombo, Sri Lanka

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ABSTRACT

Envenomation by hump-nosed pit viper (*Hypnale hypnale*, Hh) in Sri Lanka has caused significant morbidity and mortality, attributed to 35% of total venomous snakebites. In Southwestern India (Kerala), *H. hypnale* was increasingly identified as a dangerous and common source of envenomation, second to the Russell's viper but ahead of the cobra bites. Unfortunately, there is still no specific antivenom to date. This study aims to investigate the immunological properties of the venom and to assess the feasibility of specific Hh antivenom production as well as the development of a diagnostic assay. Hh venom elicited satisfactory titers of anti-Hh IgG in rabbits after 3rd immunization. The anti-Hh IgG, isolated with caprylic acid precipitation method, was effective in neutralizing the venom lethality (potency = 48 LD₅₀ per ml IgG) as well as its procoagulant, hemorrhagic and necrotic effects, indicating the possibility to produce the specific antivenom using the common immunization regime. Cross-reactivity studies using indirect ELISA showed that anti-Hh IgG cross-reacted extensively with several Asiatic crotalid venoms, particularly that of *Calloselasma rhodostoma* (73.6%), presumably due to the presence of venom antigens common to both snakes. Levels of immunological cross-reactivity were vastly reduced with double-sandwich ELISA. Further work demonstrated that the assay was able to distinguish and quantify venoms of *H. hypnale*, *Daboia russelii* and *Echis carinatus sinhalensis* (three common local viperid) used to spike human sera at various concentrations. The assay hence may be a useful investigating tool for diagnosing biting species and studying the time course profile of venom concentrations in blood.

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1. Introduction

The Merrem's hump-nosed pit viper, *Hypnale hypnale* is a medically important venomous snake in Sri Lanka and southwestern coast of India (de Silva et al., 1994; Warrell, 1995; Joseph et al., 2007; Ariaratnam et al., 2008). The hump-nosed pit viper bite has been recorded as the major cause of venomous snake bites in Sri Lanka (35% of total venomous bites, Ariaratnam et al., 2008), and being second to the Russell's viper but ahead of the cobra bite as a major cause of envenoming in central Kerala, India (Joseph et al., 2007). Envenomed victims often develop debilitating localized features, e.g. pain, edema, hemorrhagic blistering and necrosis; while in worse scenarios, potentially fatal systemic envenoming ensues with predominant features of hemostatic dysfunction that includes intravascular coagulopathy and spontaneous systemic bleeding. An infrequent but fatal complication, acute kidney injury, had been reported as well (Joseph et al., 2007; Ariaratnam

et al., 2008), and as described by Sitprija (2008), such complication is likely secondary to consumptive coagulopathy, mediated through the venom procoagulant and fibrinolytic actions. Despite its high prevalence and severe sequelae, there is still no specific antivenom to date for the treatment of *H. hypnale* envenomation (Ariaratnam et al., 2008). Nevertheless, Tan et al. (2011a) showed that two commercial antivenoms (monovalent and polyvalent) raised against Malayan pit viper (*Calloselasma rhodostoma*) venom effectively neutralized the venom lethality as well as its procoagulant, hemorrhagic and necrotic toxicities in mice. Such paraspecific venom neutralization was possible due to the presence of similar antigenic properties between the venoms of *H. hypnale* and *C. rhodostoma*, between which close phylogenetic relationships had been established (Parkinson et al., 1997; Vidal and Lecointre, 1998).

Snake envenomation, known for its neglected status, faces various global pertinent challenges including that for antivenom production and supply (Gutiérrez et al., 2006; WHO, 2009). In snake envenomation, serotherapy, i.e. administration of antivenom is the only definite therapeutic treatment (Chippaux and Goyffon, 1998). In our previous study, the Malayan pit viper antivenom has been suggested for therapeutic use in endemic areas where antidotes

* Corresponding author. Tel.: +60 3 79674951; fax: +60 3 79674791.

E-mail addresses: tanchoochock@gmail.com, tanch@um.edu.my (C.H. Tan).

are urgently needed to abate the envenoming crisis in view of its capability of cross-neutralizing *H. hypnale* venom. However, the antivenom has yet to be assessed for its clinical efficacy in human patients. We also presume that in paraspecific protection, though effective, not all toxins of the *H. hypnale* venom will be neutralized at optimum by a heterologous antivenom. Conceptually, production of specific antivenom using the homologous *H. hypnale* venom is therefore essential and warranted; and long-term efforts should be undertaken for this industry. To apply such therapeutic principle, two pertinent issues need to be addressed: (a) the antigenicity of the venom and the potency of antibodies produced and (b) the identification of biting species. *H. hypnale* has been misidentified especially as *Echis carinatus* (saw-scaled viper), or occasionally as *Daboia russelii* (Russell's viper)—two common biting snakes in those areas, hence complicating the clinical management (Joseph et al., 2007; Ariaratnam et al., 2009). Development of a diagnostic tool for accurate identification of biting species and in particular one that is able to distinguish *H. hypnale* bite from *E. carinatus* and *D. russelii* bites is therefore essential for meaningful clinical management.

In this work, the immune response elicited by *H. hypnale* venom was investigated in rabbits, and the monospecific antisera as well as the immunoglobulins (IgG) elicited by the venom antigens were assessed for venom-neutralizing potencies. The antibody cross-reactivities with several other venoms, as well as cross-reactivities of Malayan pit viper antivenoms with *H. hypnale* venom were also investigated on ELISA. The feasibility of ELISA developed as a tool for biting species identification and venom quantitation was also examined using human sera spiked with the venom.

2. Materials and methods

2.1. Venoms and antivenoms

H. hypnale venom was a pooled sample obtained from the milking of >10 adult snakes captured in Sri Lanka (Gamapha, Kelaniya, Avissawela, Colombo regions). The snakes were kept at the serpentarium at the University of Colombo, Sri Lanka and were identified by Anslem de Silva, an expert herpetologist. *D. russelii* and *Echis carinatus sinhalensis* venoms were obtained from the same source and the snakes were captured in Sri Lanka (Anuradhapura, Ratnapura, Galle, Colombo regions for *D. russelii*; and Mannar for *E. carinatus sinhalensis*). All other venoms were purchased from Latoxan (France), with exceptions of *Agkistrodon piscivorus leucostoma*, *Crotalus atrox* from Sigma (USA); *Crotalus viridis* from Miami Serpentarium (USA); *Popeia popeiorum*, *Viridovipera stejnegeri*, *Bothrops asper* from Ventoxin (USA); *Dendroaspis angusticeps*, *Pseudonaja textilis*, *Oxyuranus microlepidotus* from Venom Supplies (South Australia); *Enhydryna schistosa* from Penang Snake Venom Institute (Malaysia). Monovalent Malayan pit viper antivenom (MPVA) (Batch no. CR00909, exp. date 2/11/2014) and Hemato polyvalent antivenom (HPA) (Batch no. HP00108, exp. date 6/11/2013) were supplied by Queen Saobhva Memorial Institute, Thai Red Cross Society (TRCS).

2.2. Animals and reagents

New Zealand white rabbits and albino mice (ICR strains) were supplied by the Laboratory Animal Centre, Faculty of Medicine, University of Malaya. The animals were handled according to the guidelines given by CIOMS on animal experimentation (Howard-Jones, 1995). Human sera were a pooled sample from six healthy volunteer donors, where consent had been obtained. Ninety-six-well flat bottom microtiter plates (Nunc, Denmark), goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad Laboratories, USA), PBS-Tween 20 (Sigma, USA), ortho-phenylenediamine

(OPD) (Sigma, USA), caprylic acid (Sigma, USA), HiTrap™ Protein A-Sepharose 5-ml column (GE Healthcare, Sweden), and Vivaspın™ centrifugal concentrators (Sartorius Stedim Biotech, Germany) were purchased from the respective manufacturers. All other chemicals and reagents utilized were of analytical grade (Sigma, USA).

2.3. Immunization

Rabbits ($n = 3$) of 2 kg each were initially immunized intramuscularly over the back with *H. hypnale* venom (50 µg in 0.5 ml PBS, pH 7.4) emulsified in 0.5 ml complete Freund's adjuvant, followed by three other intramuscular doses of 100 µg in 0.5 ml PBS emulsified in 0.5 ml incomplete Freund's adjuvant at fortnightly intervals. The antibody titer levels were monitored by indirect ELISA, every two weeks following each injection.

Using the same immunization protocols, *D. russelii* and *E. carinatus sinhalensis* venoms were used to raise antibodies from rabbits ($n = 2$ and $n = 1$, respectively) for double-sandwich ELISA.

2.4. Purification of immunoglobulins G (IgG)

Antisera showing highest antibody titer (as monitored by ELISA) were collected from rabbits by cardiac puncture under anesthesia. The purification of anti-*H. hypnale* IgG was then carried out using two approaches: (A) by affinity chromatography using Protein A column and (B) by protein precipitation using caprylic acid. In (A), the sera were desalted by Sephadex G-25 gel filtration column to yield the serum protein fractions which were subsequently pooled and freeze-dried. The IgG purification was carried out as described by Hudson and Hay (1980) with slight modifications. One hundred milligrams freeze-dried serum proteins was dissolved in 1 ml 20 mM sodium phosphate (pH 8.0), and applied to the HiTrap™ Protein A-Sepharose gel affinity column connected to Micro Tube Pump MP-3 (Tokyo Rikakidai Co.). Washing of unbound components began with 20 mM sodium phosphate, followed by elution with 0.1 M citric acid (pH 3.0 in 1 M Tris-HCl pH 9.0), at flow rate of 5 ml/min. The eluted IgG were then pooled, desalted and the lyophilized powder was stored at -20°C . The purity of isolated IgG was validated on SDS-polyacrylamide gel electrophoresis according to Laemmli (1970) (data not shown). The isolated IgG were kept at -20°C for use in double-sandwich ELISA and for horseradish peroxidase conjugation. In (B), the procedure was carried out according to Rojas et al. (1994). In brief, caprylic acid was mixed with 10 ml anti-Hh sera to a final concentration of 5% at pH 6. Following vigorous stirring for 1 h at room temperature, the mixture was centrifuged at $10,000 \times g \times 10$ min, and the supernatant was collected, diafiltered, then lyophilized. The product was kept at -20°C for later use in venom neutralization studies.

Isolation of anti-*D. russelii* and anti-*E. carinatus sinhalensis* IgG was performed using the Protein A affinity chromatography method, as described above.

2.5. Conjugation of IgG with horseradish peroxidase

The assay was performed according to Tijssen (1985). Four milligrams horseradish peroxidase (HRP) was dissolved in 1.0 ml of distilled water and 0.2 ml of freshly prepared 0.1 M sodium periodate was added and stirred. The resulting HRP-aldehyde was dialyzed against 1 mM sodium acetate buffer (pH 4.4) at 4°C for several changes of buffer to reduce auto-conjugation. The pH was then raised to approximately 9.0–9.5 by adding 20 µl of 0.2 M sodium carbonate buffer (pH 9.5). Eight milligrams of IgG, dissolved in 1 ml of 0.01 M sodium carbonate buffer (pH 9.5), was added immediately. The mixture was stirred for 2 h at room temperature, followed by adding 100 µl freshly made sodium borohydride

solution (4 mg/ml in distilled water) to reduce the Schiff's base. After 2 h incubation at 4 °C, the IgG–HRP conjugate was filtered for buffer exchange with PBS (pH 7.2) at 4 °C, aliquoted and stored at –20 °C.

2.6. Indirect ELISA for determination of antibodies

Microtiter wells were each coated with 100 µl venom of *H. hypnale*, *D. russelii* or *E. carinatus sinhaleyus*, respectively (100 ng/ml in carbonate–bicarbonate buffer, pH 9.6) overnight at 4 °C. After washing with 100 µl PBS–Tween 20 for four times, each well was incubated with 100 µl serum samples diluted 1:400 in PBS–Tween 20, pH 7.4, for 1 h at room temperature. Following another cycle of washing, goat anti-rabbit IgG–HRP in PBS–Tween 20 (1:3000 dilution made up to 100 µl) was added and left for 1 h at room temperature. The HRP activity was commenced after washing by incubation with substrate o-phenylenediamine (OPD, 0.4 mg/ml and 0.006% H₂O₂ in 0.1 M citrate-phosphate, pH 5) for 30 min at room temperature in dark. The reaction was terminated by the addition of 50 µl of 12.5% H₂SO₄ and absorbance was read at 492 nm with a microplate reader (SUNRISE-TECAN Type Touch Screen F039300). All experiments were performed in triplicates.

2.7. Double-sandwich ELISA for detection of venom antigen

Microtiter wells were each coated with 100 µl affinity-purified rabbit anti-venom IgG (4 µg/ml for anti-*H. hypnale*; 5 µg/ml for anti-*D. russelii* and anti-*E. carinatus sinhaleyus*, respectively, as determined by chequerboard titration) dissolved in carbonate–bicarbonate buffer, pH 9.6, by incubating overnight at 4 °C. After washing with 100 µl PBS–Tween 20 for four times, each well was incubated for 2 h at room temperature with 100 µl venom solution (100 ng/ml) dissolved in PBS–Tween 20, or in human sera diluted with PBS–Tween 20 (1:10 dilution made up to 100 µl). Following another cycle of washing, rabbit anti-venom IgG–HRP conjugate, freshly diluted to 100 µl (1:200 for anti-*H. hypnale*; 1:600 for anti-*E. carinatus sinhaleyus*; 1:800 for anti-*D. russelii*, determined by chequerboard titration) was dispensed into each well and left for another 2 h at room temperature. The HRP activity was initiated after washing by incubation with 100 µl substrate OPD (0.4 mg/ml and 0.006% H₂O₂ in 0.1 M citrate-phosphate, pH 5) for 30 min at room temperature in dark. The reaction was terminated by the addition of 50 µl of 12.5% H₂SO₄ and the absorbance was read at 492 nm with a microplate reader (SUNRISE-TECAN Type Touch Screen F039300). All experiments were performed in triplicates.

2.8. Cross-reactivity of anti-*H. hypnale* (anti-Hh) sera and anti-Hh IgG with various venoms

Venoms of various species (100 µl of 100 ng/ml solution) were coated on microtiter walls by overnight incubation. Antisera (1:400) from the final (4th) immunization and purified IgG (1:500), diluted in PBS–Tween 20 (for both, made up to 100 µl), were each incubated with the precoated venoms then for 1 h and the cross-reactivities were determined using indirect ELISA. For double sandwich ELISA, anti-Hh IgG (100 µl of 4 µg/ml solution) was coated on microtiter wells by overnight incubation. 100 µl venoms of various species (100 ng/ml solutions) were used as samples to incubate with the precoated IgG for 2 h, and the cross-reactivities were then determined. ELISA cross-reactivities were expressed in mean percentage ± SEM of the absorbance with reference to reaction between anti-Hh sera or IgG with *H. hypnale* venom.

2.9. ELISA cross-reactivity of *H. hypnale* venom with *C. rhodostoma* monovalent and polyvalent antivenoms

For indirect ELISA, 100 µl *H. hypnale* venom (100 ng/ml) was used to coat the microtiter wells, of which after washing were incubated with 100 µl antivenoms (MPVA or HPA, with 1:5000 dilution in PBS–Tween 20 made up to 100 µl). Goat anti-equine IgG–HRP conjugate (1:8000 dilution in PBS–Tween 20 made up to 100 µl) was used as secondary antibody to which the HRP activity on OPD was then measured. A hundred microliter *C. rhodostoma* venom (100 ng/ml) coated on each microtiter well was used as the positive control, where homologous venom neutralization has been established in murine model (Tan et al., 2011a); while 100 µl *Naja naja* venom (100 ng/ml) coated on each microtiter well served as the negative control, where no in vitro cross-neutralization has been observed (data not shown).

The study was repeated with double-sandwich ELISA, where both coating and primary antibodies were replaced by antivenoms (MPVA or HPA) in 100 µl coating buffer or PBS–Tween 20 (1:5000 dilution made up to 100 µl) accordingly. Goat anti-equine IgG–HRP conjugates (1:8000 dilution in PBS–Tween 20 made up to 100 µl) served as secondary antibodies to which HRP activity on OPD was measured then. The samples consisted of 100 µl venoms (100 ng/ml) of *H. hypnale*, *C. rhodostoma* (positive control) and *N. naja* (negative control). The cross-reactivities were expressed in mean percentage ± SEM of the absorbance with reference to the controls.

2.10. Detection and quantitation of venom in human sera

H. hypnale, *E. carinatus sinhaleyus* and *D. russelii* venoms were respectively dissolved in a 1:10 mixture of human serum and PBS–Tween 20, subsequently diluted with the mixture to a series of known concentrations (0–100 ng/ml). These then served as the samples for the double-sandwich ELISA described in Section 2.7, under each optimal working condition as predetermined. The absorbance values were then plotted against the venom concentrations. Limit of detection (LoD) and limit of quantitation (LoQ) for venom detected in human sera were calculated with the LoD defined as three times (3×) the standard deviation of the blank, and the LoQ defined as ten times (10×) the standard deviation of the blank, with the blank being serum without venom antigens.

2.11. Determination of lethality, hemorrhagic, procoagulant and necrotic activities

The values of intravenous median lethal dose (LD₅₀) of Hh venom in mice, minimum hemorrhagic dose (MHD, the dose that caused a hemorrhagic lesion diameter of 10 mm), minimum coagulant dose (MCD, the minimal dose that clots a standard solution of bovine fibrinogen in 60 s), and the minimum necrotic dose (MND, the minimal dose that induces a necrotic lesion with a diameter of 5 mm) were based on previous determinations from the same laboratory reported by Tan et al. (2011a). These values were used for the in vitro neutralization studies (Section 2.12).

2.12. In vitro neutralization of the venom lethality, hemorrhagic, procoagulant and necrotic activities by anti-Hh sera and IgG

These were carried out as modified from Ramos-Cerrillo et al. (2008) and Tan et al. (2011a). Challenge doses for lethality, procoagulant, hemorrhagic and necrotic effects were respectively 2.5 LD₅₀ (for antisera), 5 LD₅₀ (for IgG), 2 MCD, 2 MHD and 2.5 MND. In brief, the venom was mixed thoroughly with various dilutions of antisera or IgG solution (made up to 50 µl for intradermal injections; and 200 µl for intravenous injections). The IgG dilution was

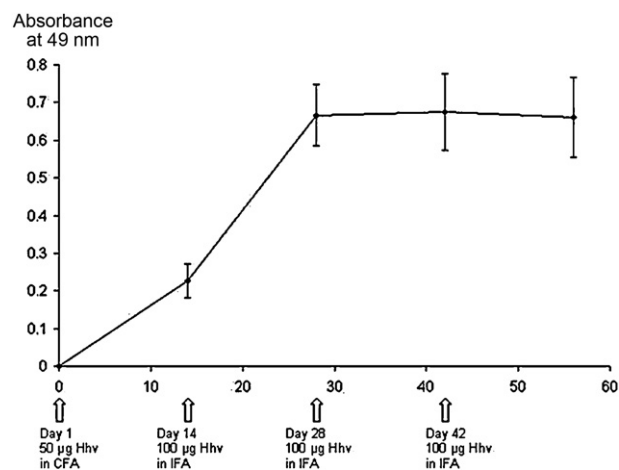


Fig. 1. Immune response elicited in three rabbits monitored by indirect ELISA following a fortnightly immunization schedule. Arrows show the days of immunization with contents of injection: Hhv: *H. hypnale* venom; CFA: complete Freund's adjuvant; IFA: incomplete Freund's adjuvant. Values were mean absorbance \pm SEM.

made according to MPVA/HPA instruction manual where 1 vial of ~1 g antivenom is to be reconstituted in 10 ml solution. The mixtures of venom and antisera or IgG were then incubated at 37 °C for 30 min with gentle shaking, following which tests for the corresponding toxic activities were carried out. The effective doses (ED or ED₅₀) were calculated as described by Tan et al. (2011a). In addition, potency of the antivenom, *P*, defined as the amount of venom (expressed in number of median lethal doses) that is completely neutralized per unit volume of antivenom, was calculated according to the European Directorate for the Quality of Medicines (1997) and Araujo et al. (2008).

2.13. Statistical analysis

The results for procoagulant, hemorrhagic and necrotic activities as well as cross-reactivities are presented as mean \pm SEM, while the variability of lethality assays is expressed as 95% confidence intervals (C.I.). The significance of the differences of the means was determined by Student's *t*-test or ANOVA test. The ED₅₀ (median effective dose) and the 95% confidence intervals (C.I.) were calculated using the probit analysis method of Finney (1952). Statistical analyses were carried out using the Biostat Analysis software.

3. Results and discussion

3.1. Immunization and antisera production

The antigenicity of *H. hypnale* venom was expressed with a steady rise of titer level following the primary immunization (Fig. 1), indicating that the venom was sufficiently antigenic to generate a good immune response in the animals. Viperid venoms are known to be qualitatively and quantitatively different from many elapid venoms, with the predominant presence of high molecular weight components especially hydrolytic enzymes, e.g. serine proteases and metalloproteases (Mackessy, 2010; Serrano et al., 2005). *H. hypnale* venom has been shown to exhibit various enzymatic activities, notably the thrombin-like enzyme, proteolytic enzyme, hyaluronidase, L-amino acid oxidase and phospholipase A₂ (Tan et al., 2011b). These toxic protein components are medium to large molecules (molecular weights of 14–28 kDa and above) which generally elicit greater antibody production, as immunogenicity is known to increase with the size of the antigen (Harlow and Lane, 1988). The use of adjuvant in the immunization scheme also served to augment the antigen complexity, hence enhancing the immune

response and antibody production (Herbert, 1978). In Fig. 1, the antibody titer level rose steadily toward the third immunization, following which it plateaued on subsequent booster. The final level upon completion of the immunization was noted to be slightly lower than the previous one but the difference was statistically insignificant ($p > 0.05$). Plateauing of the antibody titer level implied that the immune response was mature and the antibodies produced were essentially immunoglobulins G from the secondary response. The results suggested that in the production of antivenom against *H. hypnale* venom, a short duration (3 immunizations in total) may be adequate using the protocol, hence reducing the amount of venom required for antivenom production. This is an important consideration as the supply of *H. hypnale* venom is always limited due to the tiny body size of the snake (Joseph et al., 2007; Tan et al., 2011b).

The antisera production for *D. russelii* and *E. carinatus sinhaleys* venoms generally followed the similar pattern for *H. hypnale* venom, where the antibody titers rose toward the third immunization, following which a plateau was observed (data not shown).

3.2. Neutralization of the lethal and toxin effects of *H. hypnale* venom with anti-Hh sera and IgG

The antisera produced by immunization with *H. hypnale* venom in rabbits are effective in neutralizing the lethality of the venom with an ED₅₀ of 97.6 µl (95% C.I. = 46.6–187.2 µl) when tested against 2.5 LD₅₀ in mice. Preliminary tests with 5 LD₅₀, unfortunately, killed most mice despite the animals receiving maximal dose of 200 µl antisera, a phenomenon likely explained by the fact that venom–antivenom dose–response does not necessarily follow linearity at higher doses, which was also observed by Bogarin et al. (2000). The IgG isolated using caprylic acid precipitation, a common and effective way of yielding IgG in large-scale antivenom manufacturing (Rojas et al., 1994; Raweerith and Ratanabanangkoon, 2003; Khomvilai, 2008), were nonetheless effective in neutralizing the lethal effect of 5 LD₅₀ with an ED₅₀ of 83.1 µl (40.9–176.8 µl). Caprylic acid precipitation method was used in this study as to mimic the production of MPVA and HPA by the Thai Red Cross Society from hyperimmune equine sera. In order to standardize the comparison of neutralization efficacy on lethality and to avoid ambiguity using ED₅₀ alone, the parameter 'potency', *P*, was adopted as it is independent of the number of LD₅₀ used in challenge (Morais et al., 2010). Table 1 shows that the potency of the anti-Hh IgG (48 LD₅₀ per ml) was apparently higher than that of anti-Hh sera (by 3-fold), and only slightly lower than the MPVA potency (57 LD₅₀ per ml), but much less than that of HPA (96 LD₅₀ per ml).

Table 1

Neutralization of the lethal effect of *Hypnale hypnale* venom by the monovalent Malayan pit viper antivenom, Hemato polyvalent antivenom, anti-*Hypnale hypnale* sera and anti-*Hypnale hypnale* IgG.

Antivenom	ED ₅₀ (µl antivenom/challenge dose)	Potency, <i>P</i> (LD ₅₀ per ml)
Monovalent Malayan pit viper antivenom (MPVA)	70.71 µl ^a (33.7–148.4)	57
Hemato polyvalent antivenom (HPA)	41.53 µl ^a (20.4–88.4)	96
Anti- <i>Hypnale hypnale</i> sera	97.59 µl ^b (46.6–187.2)	15
Anti- <i>Hypnale hypnale</i> IgG	83.10 µl ^a (40.9–176.8)	48

^a Venom challenge dose = 5 LD₅₀.

^b Venom challenge dose = 2.5 LD₅₀.

The i.v. LD₅₀ of *H. hypnale* venom (0.9 µg/g), ED₅₀ of MPVA and HPA were based on Tan et al. (2011a).

Table 2

Neutralization of the procoagulant, hemorrhagic and necrotic activities of *Hypnale hypnale* venom by the monovalent Malayan pit viper antivenom, Hemato polyvalent antivenom, anti-*Hypnale hypnale* sera and anti-*Hypnale hypnale* IgG.

Toxic activity	Minimum dose	ED or ED ₅₀ (MPVA) (μl/mg)	ED or ED ₅₀ (HPA) (μl/mg)	ED or ED ₅₀ (anti-Hh sera) (μl/mg)	ED or ED ₅₀ (anti-Hh IgG) (μl/mg)
Procoagulant	56.2 ± 1.3 μg/ml	432.1 ± 10.9	114.2 ± 2.7	644.1 ± 8.1	556.36 ± 12
Hemorrhagic	10.5 ± 0.2 μg	472.3 ± 13.9	67.4 ± 5.1	781.00 ± 11.4	618.50 ± 12
Necrotic	39.3 ± 1.6 μg	612.6 ± 17.5	53.8 ± 0.5	646.0 ± 20.5	615.59 ± 11

The minimal doses of procoagulant, hemorrhagic and necrotic activities, as well as the effective doses of MPVA and HPA were based on Tan et al. (2011a). Values were expressed as mean ± SEM. MPVA: Malayan pit viper antivenom; HPA: Hemato polyvalent antivenom; anti-Hh: anti-*Hypnale hypnale*.

The anti-Hh sera and IgG were also effective against the procoagulant, hemorrhagic and necrotic activities of the venom (Table 2), with the anti-Hh IgG being significantly more potent than the antisera ($p < 0.05$). However, the effective doses of anti-Hh IgG were significantly higher (less potency) compared to those of HPA ($p < 0.05$), a finding in line with the result from lethality neutralization test. The IgG was also less potent than MPVA in neutralizing the procoagulant and hemorrhagic effects, but not the necrotic effect, where both were equally effective. In this study, nephrotoxicity (a severe complication of *H. hypnale* envenoming although occurs infrequently) was not assessed due to the inconsistent occurrence of acute kidney injury (unpublished observation in a rodent model), while limited venom supply was insufficient to carry out further exploration. A sublethal dose (1/3 intramuscular LD₅₀) of the venom did not induce direct kidney damage from our experience (Tan et al., 2011b), and such renal pathology may only be manifested in the context of severe consumptive coagulopathy in *H. hypnale* envenomation (Sitprija, 2008). As nephrotoxicity is the complication that causes most death, the ability of the antibody to confer protection against lethality could be tentatively explained by its neutralizing action on coagulopathy, hence preempting the development of acute kidney injury.

Generally, in neutralization of all prominent toxic activities including lethal effect, the anti-Hh IgG was effective but appeared less potent when compared to the Hemato polyvalent antivenom (HPA), suggesting that HPA at a given volume contained higher fractions of IgG domains which could recognize and bind to the *H. hypnale* venom toxin antigens. This is either due to the additional treatment during HPA manufacturing, i.e. pepsin digestion of the antibodies from hyperimmunized horses yielding concentrated (90%) F(ab')₂ fragments (Khomvilai, 2008), or the antigenic properties of components from *D. siamensis* and *C. albolabris* venoms used together with *C. rhodostoma* venom as immunogens in the production of HPA. This is supported by the observation that anti-*H. hypnale* IgG cross-reacted substantially with *C. albolabris* venom in both indirect and double-sandwich ELISA (see below and Table 3). The efficacy of anti-Hh IgG in neutralizing the procoagulant and hemorrhagic activities was also slightly lower than that of MPVA, which also had undergone pepsin digestion of its antibody. At this stage we were unable to test the various parameters to optimize caprylic acid precipitation and pepsin digestion as the amount of antisera/IgG collected were limited due to the use of small animals (rabbits) for antibody production. However, the results implied that the anti-Hh sera neutralizing capacity for venom toxicity could be improved by caprylic acid precipitation to yield the IgG. Pepsin digestion, initially used to remove allergenic Fc of heterologous antibody (Pope, 1939), should also be considered in future anti-Hh antivenom production for enhancing the therapeutic potency. The potential application of anti-Hh IgG can also be further explored in future for the venoms of *Hypnale zara* and *Hypnale nepa* (or *H. walli*, a junior synonym): two other species of *Hypnale* genus recognized currently (Maduwage et al., 2009). The venoms of all three species were shown to exhibit similar reversed-phase chromatographic pattern and toxic properties (Maduwage

et al., 2011). The envenoming by these two species is however far more infrequent than that by *H. hypnale*, which is responsible for most of the reported severe clinical problems (C.A. Gnanathasan, personal communication, 26 June 2011).

While the Malayan pit viper polyvalent antivenom (HPA) has been proposed for use in *H. hypnale* envenoming due to its medical urgency (C.A. Gnanathasan, personal communication, 26 June 2011), the production and refinement of an *H. hypnale*-specific antivenom is nonetheless the therapeutic of choice in the long term. In view of the suboptimal socioeconomic and healthcare system in the developing country, instead of producing a new monovalent antivenom, we suggest that *H. hypnale* venom be incorporated into the immunizing regimen for existing polyvalent antivenoms used in the country. This however requires further efforts in fine-tuning the optimization for antivenom efficacy, especially on a large-scale production; hence it represents a research area beyond academic exercise that awaits global partnership with supports to overcome the various challenges ahead (Gutiérrez et al., 2006).

3.3. ELISA cross-reactivities of anti-Hh sera and IgG with various venoms

Cross-reactivities of the anti-Hh antisera with various venoms on indirect ELISA were extensive: most prominent with Asiatic Crotalinae venoms, moderate with Viperinae venoms, and least with Elapidae and Hydrophiidae venoms (Table 3). ELISA non-specific binding by antisera was effectively reduced by replacing the binding antisera with affinity-purified anti-Hh IgG. Affinity chromatography is known to give a highly purified product (Kukongviriyapan et al., 1982; Carroll et al., 1992), hence minimizing false positive ELISA signals attributable to various components in the crude sera. The highest cross-reactivity of the IgG was observed with *C. rhodostoma* venom (73.6%), followed by the venoms of several Old World crotalids, with exceptionally low value for *Tropidolaemus wagleri* venom—this was not surprising as the venom is known to be quite atypical among the crotalids' whereby it does not exhibit hemorrhagic activity, is feebly procoagulant, contains unique phospholipases A₂, and is rich in low molecular weight proteins (Tan and Tan, 1989; Wang et al., 1999). Based on the extent of the cross-reactivities, *H. hypnale* venom and *C. rhodostoma* venom appear to be the most similar antigenically, and this is likely due to their very close phylogenetic relatedness despite distant geographical distribution (Parkinson et al., 1997). Observations where immunological properties of venom proteins supported snake taxonomy and systematics derived from mitochondrial DNA analysis had been reported in the case of *Trimeresurus* Complex (Tan, 2010). In this study, the cross-reactivities generally reduced with the increased distance of the snakes phylogenetic relationships, and were practically nil for venoms of Elapidae snakes. Generally, the cross-reactivities between the anti-Hh IgG with Crotalinae venoms appeared much stronger than that with the Viperinae and Elapidae venoms, suggesting that there are antigenically similar toxin structures conserved among the Crotalinae snakes venoms.

Table 3ELISA cross-reactivities of the anti-*Hypnale hypnale* sera and anti-*Hypnale hypnale* IgG with various venoms, using indirect or double-sandwich ELISA.

Venom	Test					
	Indirect ELISA with antisera		Indirect ELISA with IgG		Double-sandwich ELISA with IgG	
	Cross reactivity (%)	±SEM (%)	Cross reactivity (%)	±SEM (%)	Cross reactivity (%)	±SEM (%)
Basal crotalids						
<i>Hypnale hypnale</i>	100.0	0.0	100.0	0.0	100.0	0.0
<i>Calloselasma rhodostoma</i>	90.0	1.0	73.6	0.1	11.8	1.1
Old World, Asiatic crotalids						
<i>Protobothrops flavoviridis</i>	91.4	1.7	42.1	1.4	10.4	0.8
<i>Protobothrops tokarensis</i>	87.1	0.2	50.9	4.3	6.5	1.0
<i>Viridovipera stejnegeri</i>	82.6	0.9	61.4	0.1	9.9	2.0
<i>Popeia popeiorum</i>	82.6	0.4	27.5	1.6	6.4	1.7
<i>Cryptelytrops purpureomaculatus</i>	58.0	1.4	35.0	0.5	7.6	3.8
<i>Cryptelytrops albolabris</i>	76.5	0.6	40.2	1.2	2.1	2.1
<i>Tropidolaemus wagleri</i>	71.0	3.8	0.5	0.5	0.4	0.1
New World, invasion from Asia, Beringian dispersal crotalids						
<i>Agkistrodon piscivorus leucostoma</i>	90.1	0.3	13.0	0.8	5.4	1.3
<i>Crotalus viridis</i>	83.9	2.0	38.4	0.8	7.4	1.5
<i>Crotalus atrox</i>	60.7	1.5	24.2	0.8	6.6	2.9
<i>Bothrops asper</i>	79.8	1.4	14.8	2.3	3.1	1.2
Viperinae (Africa to Asia dispersal vipers)						
<i>Echis carinatus sinhaleys</i>	63.8	2.3	1.2	0.3	1.1	0.2
Viperinae (Eurasian dispersal)						
<i>Daboia russelli</i> (Sri Lanka)	80.4	2.7	2.5	0.4	2.0	0.4
<i>Daboia siamensis</i>	52.8	2.9	2.4	0.2	0.5	0.1
Elapids						
Elapidae (Asian dispersal)						
<i>Bungarus flaviceps</i>	21.4	0.2	0.0	0.0	0.0	0.0
<i>Bungarus candidus</i>	47.1	0.5	0.0	0.0	0.0	0.0
<i>Ophiophagus hannah</i>	13.4	2.4	0.0	0.0	0.0	0.0
<i>Naja naja</i> (Sri Lanka)	13.3	2.4	0.0	0.0	0.0	0.0
<i>Naja sputatrix</i> (Java)	14.9	1.1	0.0	0.0	0.0	0.0
Elapidae (African dispersal)						
<i>Naja nigricollis</i> (African dispersal)	17.3	0.5	0.0	0.0	0.0	0.0
<i>Dendroaspis angusticeps</i>	8.5	0.7	0.0	0.0	0.0	0.0
Elapidae (Papuo-Australian)						
<i>Pseudonaja textilis</i>	7.9	0.7	0.0	0.0	0.0	0.0
Elapidae, Subfamily Hydrophiinae (Papuo-Australasian dispersal)						
<i>Oxyuranus microlepidotus</i>	7.7	0.9	0.0	0.0	0.0	0.0
Family Hydrophiidae						
<i>Enhydrina schistosa</i>	16.6	0.3	0.0	0.0	0.0	0.0

Values were expressed as mean absorbance ± SEM. Grouping of majority of the species were done according to Wüster et al. (2008).

Double-sandwich ELISA has been known for its high specificity (Tan et al., 1993) in detection of venom antigens. It requires an antigen to be stably bound twice by a specific type of IgG: first by coating IgG, followed by HRP-labeled IgG, hence it is more stringent than direct and indirect ELISA. In this work, we demonstrated that high level of cross-reactivities of indirect ELISA was reduced markedly (>60%) using the double-sandwich ELISA, hence improving the assay specificity for *H. hypnale* venom (Table 3). In the case of anti-Hh IgG cross-reacting with the *C. rhodostoma* venom, the different magnitudes of cross-reactivities between indirect ELISA (73.6%) and double-sandwich ELISA (11.8%) suggest that the IgG, although having affinity for *C. rhodostoma* venom antigens, might have an overall lesser avidity toward them. The IgG cross-reactivities on double-sandwich ELISA were also low with the venoms of *E. carinatus sinhaleys* and *D. russelli* (two medically important vipers in Sri Lanka), hence prompting us to further investigate the potential application of double-sandwich ELISA for biting species diagnosis in *H. hypnale* envenomation (see below). Proper identification of biting snake species in Sri Lanka and Southwestern India is essential as it would ensure the appropriate therapy is instituted and avoid the use of unnecessary serotherapy that risks hypersensitivity adverse effects, especially since the biting

species had been frequently misidentified in this region (Joseph et al., 2007).

3.4. ELISA cross-reactivities of Malayan pit viper (*C. rhodostoma*) monovalent antivenom (MPVA) and Hemato polyvalent antivenom (HPA) with *H. hypnale* venom

Previous studies had shown that MPVA and, to a greater extent, the HPA, conferred paraspecific neutralization of *H. hypnale* venom (Tan et al., 2011a). Indirect ELISA showed that both antivenoms cross-reacted strongly with *H. hypnale* venom (>90%) and partially with *N. naja* venom (<50%). Using double-sandwich ELISA that has a higher degree of specificity, the cross-reactivities of MPVA and HPA with *H. hypnale* venom was shown as 90.2% and 91.5%, respectively, while that of *N. naja* venom was nil (Table 4). The findings supported the paraspecific neutralization reported earlier. The results, however, differed from the low cross-reactivity (11.8%) between anti-Hh IgG and *C. rhodostoma* venom examined on double-sandwich ELISA under the same condition. This is probably due to both higher affinity and avidity of anti-*C. rhodostoma* antibodies toward the *H. hypnale* venom, as compared to that of anti-Hh IgG toward the *C. rhodostoma* venom. We hypothesize that

Table 4

ELISA cross-reactivities between monovalent MPV antivenom and Hemato polyvalent antivenom with venoms of *Calloselasma rhodostoma*, *Hypnale hypnale* and *Naja naja*, using indirect and double-sandwich ELISA.

Venoms	Indirect ELISA				Double-sandwich ELISA			
	Cross reactivity (%) (MPVA)	±SEM (%)	Cross reactivity (%) (HPA)	±SEM (%)	Cross reactivity (%) (MPVA)	±SEM (%)	Cross reactivity (%) (HPA)	±SEM (%)
<i>Calloselasma rhodostoma</i>	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
<i>Hypnale hypnale</i>	91.7	1.2	93.6	1.9	90.2	1.7	91.5	1.1
<i>Naja naja</i>	40.3	0.7	45.9	0.4	0.0	0.0	0.0	0.0

MPVA: Malayan pit viper antivenom; HPA: Hemato polyvalent antivenom. Both antivenoms were previously noted effective in cross-neutralization of *Hypnale hypnale* venom, but totally ineffective against *Naja naja* venom. *C. rhodostoma* is the homologous species from which the antivenoms were produced; *H. hypnale* is phylogenetically closest to it, while *N. naja* is phylogenetically most distant among the three.

the anti-*C. rhodostoma* antibodies exhibit greater number of multivalencies (paratopes) that were able to recognize epitopes of *H. hypnale* venom, in contrast to anti-Hh IgG which multivalent sites are likely endowed with fewer binding sites for the antigenic epitopes of *C. rhodostoma* venom.

3.5. Detection and quantitation of venom in human sera

Using double-sandwich ELISA, the titration of *H. hypnale* venom concentrations in human sera yields a parabolic curve with a regression factor of 0.9955, within the concentration range of 0–75 ng/ml (Fig. 2a). The assay was sensitive with the limit of detection (LoD) of 2.78 ng/ml and the limit of quantitation (LoQ) of 10.88 ng/ml venom with 1:10 dilution in human serum. As *H. hypnale* is small in size (≤ 50 cm), the average dry mass of venom yield per milking is small too (average 13 mg) (Tan et al., 2011b). Given a situation where 13 mg of venom was injected into a 60-kg Asian man with plasma volume of 2.5 L (through bite on muscle and subcutaneous tissue), and the systemic absorption was estimated to be 5% (unpublished data on bioavailability), the theoretical serum concentration of venom would be close to 260 ng/ml, of which 1:10 dilution would be equivalent to 26 ng/ml and is detectable and quantifiable by the assay.

Besides, the double-sandwich ELISA demonstrated practically no cross-reactivities with the venoms of *E. carinatus* and *D. russelii*, two endemic biting snakes in the same regions which often cause misidentification for *H. hypnale*. We broadened the venom identification studies by including two separate assays for *D. russelii* and *E. carinatus sinhalensis* venoms, using double-sandwich ELISA (Fig. 2b and c). Both the assays were sensitive toward their respective homologous venoms, with the LoD and LoQ being respectively 1.64 ng/ml and 5.75 ng/ml for *E. carinatus sinhalensis*; while for *D. russelii*, 2.43 ng/ml and 9.83 ng/ml. Generally, each assay was able to distinguish the homologous venom from the other two based on a significant difference in the absorbance values at each concentration ($p < 0.05$). This suggests that the double-sandwich ELISA developed from affinity-purified anti-venom IgG has the potential to be developed into a specific diagnostic kit for viperid envenomation in the region, and this is important in clinical studies where the biting species needs to be accurately diagnosed, and the blood venom levels to be monitored. It also has the potential to improve routine clinical diagnosis and to ensure that appropriate serotherapy is instituted. Although in practice, many dubious snakebite cases were empirically treated with polyvalent antivenoms, this approach can be dangerous and is not appropriate in *H. hypnale* envenomation as the polyvalent antivenom available in Sri Lanka is

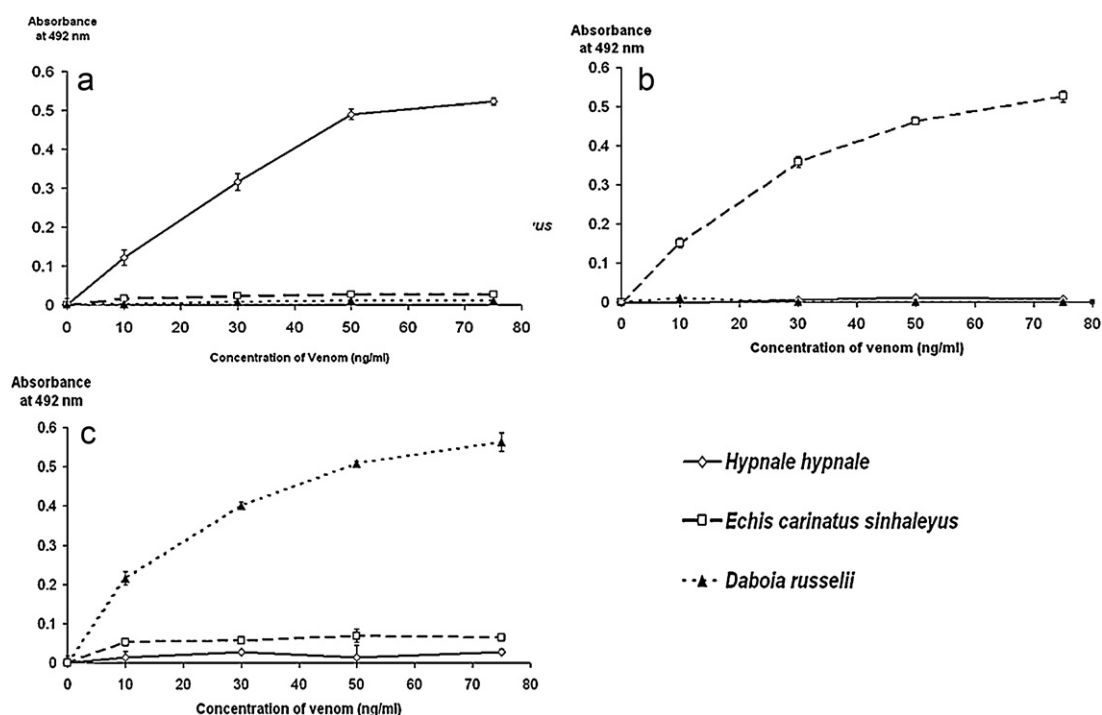


Fig. 2. Titration curve for the concentrations of venoms from three common biting snakes in Sri Lanka dissolved in human serum, with 1:10 dilution in PBS–Tween 20, on double-sandwich ELISA using IgG for *Hypnale hypnale* venom (a), *Echis carinatus sinhalensis* (b) and *Daboia russelii* (c). Values were mean absorbance \pm SEM.

only effective against *D. russelii* and *E. carinatus*, but not *H. hypnale*. In addition, the documentation of accurate biting species identity is vital for epidemiology study, an important aspect for global health sustainability; and this also provides accurate information for future research at both clinical and laboratory settings.

In highlighting the benefits of a potential 'Sri Lankan hematoxic viperids' diagnostic kit, cost is always an obstacle that should be addressed. Among the various proposed and experimental venom-screening tools, we believe that ELISA is the most economical, least technically demanding and most stable way as compared to radioisotopic assay, polymerase chain reaction or immunoblotting. A portable pocket-sized plate can be designed to have three parallel panels, each consisting two control wells (negative and positive) and two to three sample wells for venom detection based on chromogenicity. ELISA is a rather generic facility that should be available in major medical centers in the developing countries for common serology use, and in places where such facility has been equipped, costing would mainly concern consumables like buffers, IgG, enzyme and substrate, which are generally required in very small, diluted amounts. Hence, it is hoped that with international collaborative supports, such diagnostic application can be made possible at low-resource setting.

4. Conclusion

Immunological properties of *H. hypnale* venom were profiled according to potential clinical applications, where the specific IgG produced showed venom-neutralizing potency, indicating the possibility of large-scale antivenom manufacturing. Caprylic acid precipitation and pepsin digestion are steps to be considered in the production for enhancing the antivenom efficacy. Results of cross-reactivity studies were in line with the phylogenetic relationship between *C. rhodostoma* and *H. hypnale*, and supported the paraspecific cross-neutralization in our previous work. The double-sandwich ELISA is specific to distinguish among venoms of *H. hypnale*, *D. russelii* and *E. carinatus sinhaleyus*, hence the potential as a diagnostic and investigating tool in envenoming cases by these Sri Lankan hematoxic viperid snakes that are at time misidentified. This is particularly relevant to a recent progress where the paraspecific Hemato polyvalent antivenom has been proposed for use in *H. hypnale* envenoming, as the assay may serve as an investigating tool to ascertain the species diagnosis and to study the time course profile of blood venom concentrations during clinical trial.

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Short communication

Nephrotoxicity of hump-nosed pit viper (*Hypnale hypnale*) venom in mice is preventable by the paraspecific Hemato polyvalent antivenom (HPA)Choo Hock Tan^{a,*}, Nget Hong Tan^b, Si Mui Sim^a, Shin Yee Fung^b, Pailoor Jayalakshmi^c, Christeine Ariarane Gnanathan^d^a Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia^b Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia^c Department of Pathology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia^d Department of Clinical Medicine, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka

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ABSTRACT

Mice experimentally envenomed with *Hypnale hypnale* venom (1× and 1.5× LD₅₀) developed acute kidney injury (AKI) principally characterized by raised blood urea and creatinine. Prolonged blood clotting time and hemorrhage in lungs implied bleeding tendency. Pallor noted in most renal cortices was suggestive of renal ischemia secondary to consumptive coagulopathy. Intravenous infusion of Hemato polyvalent antivenom following experimental envenoming effectively prevented death and AKI in all mice, supporting its potential therapeutic use in envenoming cases.

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Bites by hump-nosed pit vipers (*Hypnale hypnale*) have been identified as a leading and potentially fatal cause of snake envenomation in Sri Lanka as well as southwestern region of India (Kerala) (Ariaratnam et al., 2008; Joseph et al., 2007). The majority (90%) of envenomed patients suffered from local tissue damages, e.g., hemorrhagic blistering and extensive tissue necrosis, while approximately 40% of them developed concurrent systemic syndrome characterized by hemostatic derangement. Acute kidney injury (AKI), a severe systemic complication associated with the bite, was responsible for most deaths with an overall fatality rate of 1.8%, even though it occurred infrequently (10% of total bite cases, $n = 302$) (de Silva et al., 1994; Ariaratnam et al., 2008). The mechanism of kidney injury by *H. hypnale* venom was postulated to be a complication of consumptive coagulopathy secondary to the venom's

procoagulant and fibrinolytic actions (Kanjanaabuch and Sitprijia, 2008), and the venom at a sublethal dose (1/3 intramuscular LD₅₀) did not appear to induce direct kidney damage in a rat model (Tan et al., 2011b). Nevertheless, in view of the venom's prominent proteolytic and cytotoxic activities (Tan et al., 2011b; Maduwage et al., 2011), a direct tissue-damaging effect on the kidney or indirect immunological responses induced by the venom at higher doses cannot be completely ruled out.

Although antivenom is the definite treatment for snakebite envenoming (Chippaux and Goyffon, 1998), there are currently no effective antivenoms clinically available to treat *H. hypnale* envenoming. A paraspecific antivenom (Hemato polyvalent antivenom, HPA, raised against venoms of Thai *Calloselasma rhodostoma*, *Cryptelytrops albolabris* and *Daboia siamensis*), previously shown effective to neutralize the venom's hematoxic, necrotic and lethal effects in mice (Tan et al., 2011a), is nonetheless a potential therapeutic treatment for the endemic medical urgency.

* Corresponding author. Tel.: +60 3 79674951; fax: +60 3 79674791.

E-mail address: tanchoochock@gmail.com (C.H. Tan).

Clinically, although most cases of AKI complicating *H. hypnale* bite were reversible with acute care (demanding dialysis), some surviving patients eventually progressed to chronic kidney disease with ensuing death within a year, simply because of the lack of affordable renal replacement therapy in a developing country that is socioeconomically constrained (Ariaratnam et al., 2008). Therefore, the antivenom therapy proposed for the treatment of *H. hypnale* bite needs to address the issue of venom nephrotoxicity in addition to its neutralization of other major toxic effects, and above all, lethality. In this study, the HPA was tested for its ability to prevent the development of AKI and death using an *in vivo* nephrotoxic animal model.

Albino mice of ICR-strain used in the current study were supplied by the Laboratory Animal Center, University of Malaya. The animals were handled according to the guidelines given by CIOMS on animal experimentation, and the experimental protocol was approved by the Animal Use and Care Committee of the institution [ethics clearance number: PM/03/03/2010/FSY(R)]. The mice were divided into one control group and two challenge groups (Group A and Group B). Mice in the control group ($n = 4$) each received 200 μ L normal saline, while mice in Group A ($n = 9$) each received *H. hypnale* venom at $1 \times$ intraperitoneal LD₅₀, and those in Group B ($n = 4$) each received the same venom at $1.5 \times$ intraperitoneal LD₅₀. The venom (a pooled sample obtained from the milking of >10 snakes captured in Gamapha, Kelaniya, Avissawela and Colombo regions of Sri Lanka) was dissolved in normal saline and the volume of injection was adjusted to 200 μ L. Both venom and vehicle (normal saline) were injected intraperitoneally. The dose used in this study was estimated from the intraperitoneal LD₅₀ value of *H. hypnale* venom in rodents (6 μ g/g) obtained in an earlier study (Tan et al., 2011b) from the same laboratory. Following injections, the mice were housed individually in standard metabolic cages with access to food and water *ad libitum*. Hourly observations on the mice's behaviors and toxic signs were charted over 48 h. Mice that showed features suggestive of impending death (e.g., labored breathing with pauses, feeble heart-beat, loss of righting reflex, inability to walk, and lack of response to manipulation) were more closely monitored until spontaneous death. Upon death, blood was collected immediately from the heart, and the major organs were then harvested for gross and microscopic examinations. At 48 h, all surviving mice were sedated with a mixture of xylazine and ketamine (0.1 and 1.0 mg, respectively, per 20 g mouse; chemicals supplied by Troy Laboratories, Australia), and blood was collected via cardiac puncture. Subsequently, the mice were euthanized by cervical dislocation, and the major organs were harvested. Blood collected in plain tubes were observed for clotting time before processing for urea and creatinine analysis. The urine samples collected throughout the study period were analyzed for specific gravity, hematuria and proteinuria using urine test strips (Roche Diagnostics, Germany). The harvested organs were fixed in 10% formalin in phosphate-buffered saline, followed by paraffin wax-embedding, sectioning of tissue, dewaxing, rehydration, and staining with hematoxylin and eosin. Kidney tissues were specifically sectioned at 1.5 μ m thin layer.

The experimental envenoming with $1 \times$ and $1.5 \times$ LD₅₀ was repeated in Group C ($n = 9$) and Group D ($n = 4$), respectively. Intervention was carried out 5 min following the venom challenge, where each mouse from both groups received an injection of 200 μ L of Hemato polyvalent antivenom (reconstituted as 1 vial of HPA: 10 mL normal saline; antivenom was supplied by Queen Saovabha Memorial Institute, Bangkok) via tail vein. Animal housing, monitoring of signs, collection of blood and urine, processing of tissues/organs of the mice and further analyses were performed essentially as described in the nephrotoxic model above. The significance of the differences in the means of the results was determined by one-way ANOVA and Tukey's post hoc test.

H. hypnale venom at median lethal dose (LD₅₀) (Group A) caused serum biochemical derangement suggestive of acute renal failure, most prominently uremia (and observably increased blood creatinine level) that affected all challenged mice (Fig. 1a). Elevated blood urea level (directly proportionate to blood urea nitrogen) generally indicates a moderate-to-severe degree of renal failure. The degree of hematuria and proteinuria correlated with the deterioration of renal function and signs of toxicity observed in mice that were dead by 24 h (Table 1). In mice challenged with $1.5 \times$ LD₅₀ (Group B), severe azotemia (with blood creatinine increased higher than urea) was observed in all mice (Fig. 1b), indicating that the renal function was further compromised. While significant proteinuria and prolonged clotting time were observed, there was however no hematuria detected, probably because glomerular involvement was not extensive in view of the short period of time to death. On autopsy, patches of pallor noted on the renal cortices were suggestive of renal ischemia, although histopathology study did not reveal obvious abnormal findings under light microscopy within the study period (48 h maximum). This suggests that electronic microscopic examinations may be necessary for investigating subcellular ultrastructural changes in the mouse model. Additional features implying possible bleeding disorder included prolonged clotting times (Table 1) and hemorrhagic spots in the lungs. Urine specific gravity was noted to be marginally higher in the challenge group with $1.5 \times$ LD₅₀ ($p = 0.04$; Table 1), a peripheral finding suggestive of pre-renal azotemia as in reduced renal perfusion secondary to altered hemodynamics.

The result indicated that death in mice experimentally envenomed with *H. hypnale* venom was associated with AKI, consistent with clinical reports (Joseph et al., 2007; Ariaratnam et al., 2008). This likely cause of death was effectively prevented by the Hemato polyvalent antivenom (HPA) in the current *in vivo* rodent model, evidenced by normalization of blood and urine biochemistry of the envenomed animals (Fig. 1a: significant amelioration of hematuria ($p \leq 0.001$), proteinuria ($p \leq 0.001$) and uremia ($p \leq 0.05$); Fig. 1b: significant amelioration of proteinuria ($p \leq 0.05$), uremia ($p \leq 0.001$) and azotemia ($p \leq 0.001$)). Moreover, in the intervened groups, urine specific gravity was improved significantly ($p \leq 0.05$), along with normal anatomy of the kidneys and lungs as well as 100% survival. The HPA neutralizing efficacies on *H. hypnale* venom has been established previously by Tan et al. (2011a): against lethality, median effective dose at 1.52 mg venom/mL;

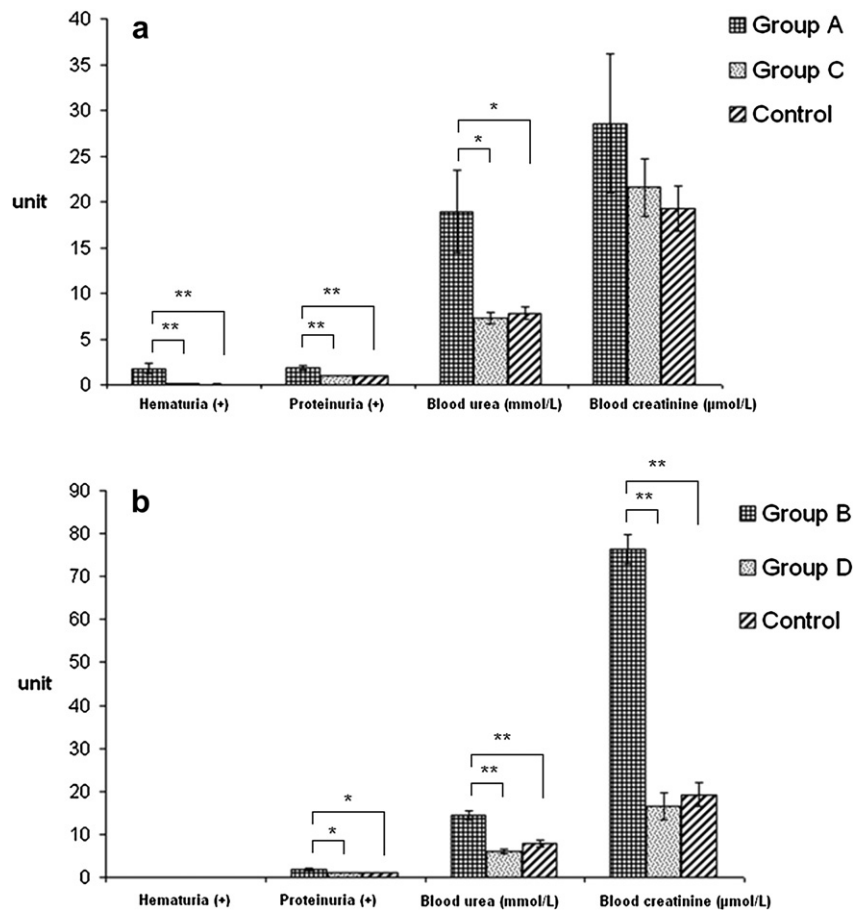


Fig. 1. Urine and blood biochemical parameters of mice: (a) Group A was challenged with $1 \times$ i.p. LD₅₀ ($n = 9$, 55% death); (b) Group B was challenged with $1.5 \times$ i.p. LD₅₀ ($n = 4$, 100% death). Group C and Group D were the intervened groups which received i.v. 200 μ L of antivenom (HPA) 5 min after envenomation for each mouse. Control was treated with normal saline instead of venom and antivenom. Data were expressed as means \pm SEM. Significant differences of mean between the challenged group and the intervened and/or control groups were indicated as * ($p \leq 0.05$) or ** ($p \leq 0.001$) over the bars, determined by one-way ANOVA and Tukey's post hoc test. No significant difference of means was noted between the intervened group and the control.

against procoagulant, hemorrhagic and necrotic effects, effective/median effective doses at 114.2, 67.4, and 53.8 μ L/mg venom, respectively. The mechanism of nephrotoxicity neutralization likely involved the HPA cross neutralizing the procoagulant and hemorrhagic effects of *H. hypnale* venom, hence preempting AKI, a complication associated with consumptive coagulopathy (Sitprija, 2008). The cross neutralization observed was also recently supported by

immunological cross reactivity study (Tan et al., 2012), which reflected similarity of antigenic properties between the venoms of *H. hypnale* and *C. rhodostoma*. However, venom-induced defibrinogenation complicating AKI is not a known feature in *C. rhodostoma* envenoming (Ho et al., 1986), hence implying subtle differences in the biochemical aspects of the procoagulant enzymes from these two crotalid venoms, possibly at the fibrinolytic

Table 1

Effects of *Hypnale hypnale* venom on urine and blood parameters of mice challenged with $1 \times$ LD₅₀ (Group A) and $1.5 \times$ LD₅₀ (Group B).

Group	Test	Urine			Blood		
		Specific gravity	Hematuria (+)	Proteinuria (+)	Clotting time (s)	Urea (mmol/L)	Creatinine (μ mol/L)
Control ($n = 4$)	Not observed	1.023 ± 0.002	0.0 ± 0.0	1.0 ± 0.0	12.5 ± 2.9	7.8 ± 0.6	19.3 ± 3.1
A ($n = 9$)	18.4 ± 2.1	1.026 ± 0.001	1.8 ± 0.6^a	1.9 ± 0.3	28.9 ± 6.5^b	19.0 ± 4.5	28.6 ± 7.6
B ($n = 4$)	7.4 ± 1.3	1.029 ± 0.001^a	0.0 ± 0.0^a	1.8 ± 0.3	45.0 ± 2.9^b	14.4 ± 1.0	76.3 ± 3.3^c

Venom was dissolved in normal saline and volume was adjusted to 200 μ L for intraperitoneal injection. Data were expressed as means \pm SEM. Fatality in Group A = 56%; Group B = 100%.

Means (for Groups A and B) indicated with letters showed significant difference from the corresponding means of control for the test, as determined by one-way ANOVA and Tukey's post hoc test: ^a $p \leq 0.05$; ^b $p \leq 0.01$; ^c $p \leq 0.001$.

mechanism. Essentially, the current findings based on *in vivo* animal experiments complement that of the cross neutralization study reported previously on the venom hematoxic and lethal effects, hence supporting the ability of HPA to confer paraspecific protection, and justifying the suggestion for its clinical trial in the future.

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Conflict of interest

None declared.

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Appendix B.**Appendix D. List of manuscripts in preparation for submission**

Tan, C. H., Sim, S. M., Gnanathanan, C. A., Fung, S. Y., & Tan, N. H. Pharmacokinetics of *Hypnale hypnale* (Hump-nosed Pit Viper) Venom in Rabbits following intravenous and intramuscular administration.

Tan, C. H., Tan, N. H., Sim, S. M., Fung, S. Y., Gnanathanan, C. A. Alterations in the Pharmacokinetics of the Venom of *Hypnale hypnale* (Hump-nosed Pit Viper) by a Parasepcific Antivenom

Tan, C. H., Tan, N. H., Sim, S. M., Fung, S. Y., Gnanathanan, C. A. Proteomic Study of the Venom of *Hypnale hypnale* (Hump-nosed Pit Viper).

Tan, C. H., Tan, N. H., Sim, S. M., Fung, S. Y., Gnanathanan, C. A. Isolation and Characterizations of a Thrombin-like Enzyme from the Venom of *Hypnale hypnale* (Hump-nosed Pit Viper).